Letters in Applied Microbiology 2004, **39**, 187–193 doi:10.1111/j.1472-765X.2004.01559.x

Comparison of primers for the detection of *Salmonella enterica* serovars using real-time PCR

A.T. Csordas[1], J.D. Barak[2] and M.J. Delwiche[1]

[1]Department of Biological and Agricultural Engineering, University of California at Davis, Davis, CA, and [2]Produce Safety and Microbiology Research Unit, USDA Agricultural Research Service, Albany, CA, USA

2004/0186: received 18 February 2004, revised 27 April 2004 and accepted 11 May 2004

ABSTRACT

A.T. CSORDAS, J.D. BARAK AND M.J. DELWICHE. 2004.

Aims: To evaluate the specificity and sensitivity of PCR primers for the detection of *Salmonella enterica* in a real-time PCR assay using pure cultures.

Methods and Results: Unenriched whole cells in sterile water were used as template for each PCR. SYBR Green dye was used for the nonspecific detection of dsDNA. The real-time PCR detection limits of five previously published primer sets used in conventional PCR applications were not below 3×10^3 CFU per reaction (rxn). A new primer set, Sen, was designed, which detected *Salm. enterica* Newport down to 6 CFU rxn^{-1} in one case, and gave an average detection limit of 35 CFU rxn^{-1} over three separate runs.

Conclusions: Primers originally designed for end-point PCR did not have adequate specificity or sensitivity compared with those specifically designed for real-time PCR.

Significance and Impact of the Study: This study emphasizes the importance of evaluating real-time PCR primer sets in pure cultures prior to testing in field samples. This study will benefit other researchers in selecting an appropriate primer set for real-time PCR detection of *Salm. enterica*.

Keywords: dissociation analysis, *invA* gene, primer dimer, SYBR Green, threshold cycle.

INTRODUCTION

Real-time PCR offers advantages over traditional PCR in that it is possible to watch the PCR reaction as it occurs, the PCR product does not have to be removed from its reaction chamber for post-PCR analysis, and many detection chemistries are available, including probe-based systems that provide assurance that the correct fragment is amplified. Primer sets that have shown *Salmonella* specificity in traditional PCR applications require evaluation in real-time PCR conditions to determine if these sets are specific and sensitive. Conventional PCR primers that have a strong tendency to form primer dimers may yield poor sensitivity results in a real-time assay. Since primer dimers are dsDNA, they will generate fluorescent signals (Higuchi *et al.* 1992) that yield false-positive results. If the primers form dimers, then at the lower target concentrations it may not be possible to distinguish between target fluorescent signals and primer dimer fluorescent signals by examining plots of relative fluorescence as a function of cycle number. In the absence of conventional PCR primers that yield adequate detection limits in real-time PCR, new primers can be designed or a probe-based system can be designed that yields fluorescence only in the presence of a specific dsDNA target.

Several real-time PCR assays have recently been developed for the detection of *Salmonella* (Hoorfar *et al.* 2000; Eyigor *et al.* 2002; Bhagwat 2004). Previous studies have shown comparisons of primers used in traditional PCR assays (Gooding and Choudary 1999; Malorny *et al.* 2003; Ziemer and Steadham 2003). The purpose of this work was to compare primer sets in a real-time PCR assay and to find a primer set that gives specific and sensitive detection of

Correspondence to: Michael Delwiche, University of California, Department of Biological and Agricultural Engineering, One Shields Ave., Davis, CA 95616, USA (e-mail: mjdelwiche@ucdavis.edu).

Salmonella enterica serovars associated with food-borne outbreaks from produce.

MATERIALS AND METHODS

Bacterial serovars and growth media

All bacteria used in this study are listed in Table 1. Bacteria were cultured on Luria-Bertani agar plates (LB, Difco/BBL, Sparks, MD, USA). Bacteria from an 18-h culture grown on LB plates were diluted in sterile water to an optical density of 0·2 at 600 nm. Serial dilutions were made in sterile water to obtain appropriate cell densities for PCR testing. Cell densities were quantified by plating 100 μl of the appropriate serial dilution on LB plates. The plates were incubated at 37°C overnight and colonies were counted.

Reagents and primers

Primer set sequences and references are shown in Table 2. All primer sets in Table 2, other than Sen, were shown to be specific for *Salmonella* in previously published research by testing with *Salmonella* and non-*Salmonella* target DNA. SYBR Green Master Mix (Applied Biosystems, Foster City, CA) reagent was used in initial experimentation with the Sal, INVA, ST, Fim, and iroB primer sets at primer concentrations of 500 nmol l^{-1}. However, further experimentation with low *Salm. enterica* cell concentrations indicated that there was a tendency for primer dimer formation. In an attempt to minimize this problem, SYBR Green Core Reagents (Applied Biosystems) were used to allow changes in the reagent concentrations. The final core reagent concentrations used in each 50 μl reaction were: 1X SYBR Green PCR Buffer, 3·0 mmol l^{-1} $MgCl_2$, 1·0 mmol l^{-1} dNTP mix with dUTP, and 1·25 U AmpliTaq Gold. In addition, 0·5 U AmpErase UNG enzyme (Applied Biosystems) was added to all Sen primer set reactions. Reduced concentrations of 250 nmol l^{-1} were tested with the Sal and INVA primer sets in sensitivity testing. All experiments with the Sen primer set were performed at primer concentrations of 250 nmol l^{-1} with the core reagents.

Mixtures of SYBR Green Master Mix or SYBR Green Core Reagents with the appropriate primer set were vortexed prior to transfer to the PCR tubes. The total volume of SYBR Green mix including primers was 30 μl (including water if necessary to adjust the volume to 30 μl). Twenty microlitres of the appropriate *Salm. enterica* serial dilution were then added to each tube to give a total

Table 1 Bacteria used for specificity testing

	Strain	Reference/source
Salm. enterica (serovar, serogroup)		
Saintpaul, B	55	SARB*
Schwarzengrund, B	96E01152C-TX-1	Inami and Moler (1999); CDHS†
Mbandaka, C_1	99A1670	CDHS
Newport, C_2	96E01153C-TX	Inami and Moler (1999); CDHS
Albany, C_3	96E01152C-TH	CDHS
Enteritidis, D_1	00A-2768	CDHS
Baildon, D_2	99A-23	CDHS
Meleagridis, E_1	96A7406	CDHS
Senftenberg, E_4	59	SARB
Rubislaw, F	54	SARB
Poona, G_1	00A3563	Barak *et al.* (2003); CDHS
Cubana, G_2	98A9878	Mohle-Boetani *et al.* (2001); CDHS
Havana, G_2	98A4399	Mohle-Boetani *et al.* (2001); CDHS
Saphra, I	97A3312	Mohle-Boetani *et al.* (1999); CDHS
Non-*Salmonella* species		
E. coli O137:H41	MW421	Wachtel *et al.* (2002)
Rahnella aquatilis	SPS2F10	Barak *et al.* (2002)
Pseudomonas putida	BM19	Barak *et al.* (2002)
Pantoea agglomerans	SPS2F1	Barak *et al.* (2002)
Erwinia chrysanthemi	3937	N. Perna‡
Klebsiella pneumonia	S48565.3	SFGH§

*SARB, *Salmonella* reference collection B.
†CDHS, California Department of Health and Human Services.
‡Nicole Perna, University of Wisconsin-Madison.
§SFGH, San Francisco General Hospital.

Table 2 Primer target, name, sequence, expected amplicon length and reference

Target fragment	Primer sets	Primer (5′ → 3′)	Product size (bp)	Reference
invA gene	Sal-3	TAT CGC CAC GTT CGG GCA A	275	Wang *et al.* (1997)
	Sal-4	TCG CAC CGT CAA AGG AAC C		
invA gene	INVA-1	ACA GTG CTC GTT TAC GAC CTG AAT	244	Chiu and Ou (1996)
	INVA-2	AGA CGA CTG GTA CTG ATC GAT AAT		
iroB gene	Primer 1	TGC GTA TTC TGT TTG TCG GTC C	606	Bäumler *et al.* (1997)
	Primer 2	TAC GTT CCC ACC ATT CTT CCC		
Random fragment	ST11	GCC AAC CAT TGC TAA ATT GGC GCA	429	Soumet *et al.* (1999)
of *Salm.* Typhimurium	ST15	GGT AGA AAT TCC CAG CGG GTA CTG G		
fimA gene	Fim1A	CCT TTC TCC ATC GTC CTG AA	85	Cohen *et al.* (1996)
	Fim2A	TGG TGT TAT CTG CCC GAC CA		
invA gene	Sen-1	TTT CAA TGG GAA CTC TGC	172	This work
	Sen-2	AAC GAC GAC CCT TCT TTT		

reaction volume of 50 μl. Negative control reactions used 20 μl of sterile water to make a total reaction volume of 50 μl.

Polymerase chain reactions were performed with a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using the following thermal cycling conditions: 94°C for 5 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. The annealing temperature was lowered to 54°C for the Sen primer set due to its lower melting temperature. Prior to the 94°C hold for 5 min, an initial step of 50°C for 2 min was added to the Sen primer set protocol to activate the AmpErase UNG enzyme. After each PCR, a dissociation profile was generated by increasing the temperature from 60 to 90°C in increments of 0·4°C over 20 min, while measuring changes in fluorescence.

Gel analysis

In addition to dissociation curve analysis, PCR amplification fragments were run in 2% agarose gels in Tris–acetate–EDTA buffer to confirm fragment size. Following the PCR, 2 μl of 6X tracking dye (Promega, Madison, WI, USA) were mixed into the 50 μl reaction volume and 10 μl of this mixture were used for gel electrophoresis. A Hi-Lo DNA ladder (Minnesota Molecular, Minneapolis, MN, USA) was used to compare fragment sizes. Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) or ethidium bromide for 10–20 min. The agarose gels were then photographed under u.v. excitation.

Primer design

Software (Primer Premier 5.0, PREMIER Biosoft International, Palo Alto, CA, USA) was used for assessment of the previously published primer sets and design of a new one. Given a particular target sequence and primer set, the software calculates the Gibbs free energy (ΔG) required for specific and nonspecific products to form, thereby allowing primers to be evaluated prior to experimentation. A large negative ΔG means that a reaction is energetically favourable and, therefore, indicates a tendency to form a dsDNA product. In addition to searching for potential primer dimers, the software can find locations outside of the target region where primers have a tendency to anneal, leading to nonspecific product formation called false priming. The *invA* gene from *Salm. enterica* serovar Typhimurium LT2 (accession number: AE008832, McClelland *et al.* 2001) was used as target DNA for software analysis of the Sal and INVA primer sets, and for designing a new primer set.

DNA sequencing

PCR products from the Sen primer set were sequenced to make certain that the target sequence was amplified. PCR products for sequencing were produced with the Sen primer set real-time PCR procedure described previously. DNA samples were purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Sequencing was performed by the Division of Biological Sciences DNA Sequencing Facility (University of California, Davis, CA, USA).

Experimental procedures

Each primer set was evaluated for its ability to amplify products from *Salm. enterica* serovars and from low initial template concentrations (sensitivity). Experimental conditions and procedures are summarized in Table 3. The Sal, INVA, iroB, ST, and Fim primer sets were tested in one experiment with the *Salm. enterica* serovars listed in Table 1 at 10^3CFU per reaction (rxn), and the Sen primer set was tested in one experiment with all bacteria shown in Table 1 at 10^3 CFU rxn^{-1}. Dissociation plot peaks and amplification fragments on agarose gels were used to verify that the

Table 3 Procedures and experimental conditions

Procedure	Primer set	[Primer] (nmol l^{-1})	PCR reagents*	Bacteria	[Bacteria] (CFU rxn^{-1})
Initial screening, traditional primers	Sal, INVA, iroB, Fim, ST	500	Master Mix	*Salm. enterica* serovars, Table 1	10^3
Sensitivity	Sal, INVA	250	Core Reagents	*Salm. enterica* Newport	10^6–10^3
Software analysis	Sal, INVA, Sen	–	–	–	–
Specificity	Sen	250	Core Reagents	All bacteria, Table 1	10^3
Sensitivity	Sen	250	Core Reagents	*Salm. enterica* Newport	10^6–10^0

*SYBR Green Master Mix or Core Reagents.

correct sequences were amplified. A primer set was considered for sensitivity testing if the initial results indicated single peaks in the dissociation plots, no peaks in the negative control dissociation plots and if agarose gel electrophoresis indicated that the correct size fragment was formed. Sensitivity testing involved three replicates per experiment using three separate experiments with each primer set. *Salm. enterica* Newport, an outbreak strain associated with produce, was used for sensitivity testing with levels ranging from 10^6 to 10^3 CFU rxn^{-1} for the Sal and INVA primer sets and approximately 10^6 to 10^0 CFU rxn^{-1} for the Sen primer set.

The detection limit of a particular primer set was determined by comparing the mean threshold cycle (C_T) value of the dilution with the mean C_T value of the negative control reactions. In the case of the Sal and INVA primer sets a one-tailed Student's *t*-test with $\alpha = 0{\cdot}05$ was performed to determine if there was a significant difference between the mean C_T values of the negative control reactions and the reactions containing the *Salm. enterica* Newport dilution of interest. Primer set detection limits were established by determining the lowest *Salm. enterica* Newport cell density mean C_T that was significantly different from the negative control.

RESULTS

Testing Sal, INVA, iroB, Fim, and ST primer sets using *Salm. enterica* serovars

The Sal, INVA, iroB, Fim and ST primer sets were tested with all *Salm. enterica* serovars shown in Table 1. Figure 1(a,b) show typical dissociation plots with each of the primer sets. The dissociation plots of the iroB and ST primer sets displayed multiple peaks with each serovar, while agarose gel electrophoresis showed a single band at the expected size in all cases. Without sequencing the PCR products, it was difficult to conclude if single or multiple products were formed with the iroB and ST primer sets. Furthermore, the iroB gel results produced faint bands and small dissociation peaks for *Salm. enterica* serovars Newport, Albany, Enteritidis and Baildon with significant amounts of primer dimer formation seen in these reactions. Thus, the iroB and ST primer sets were not used in the sensitivity experiments.

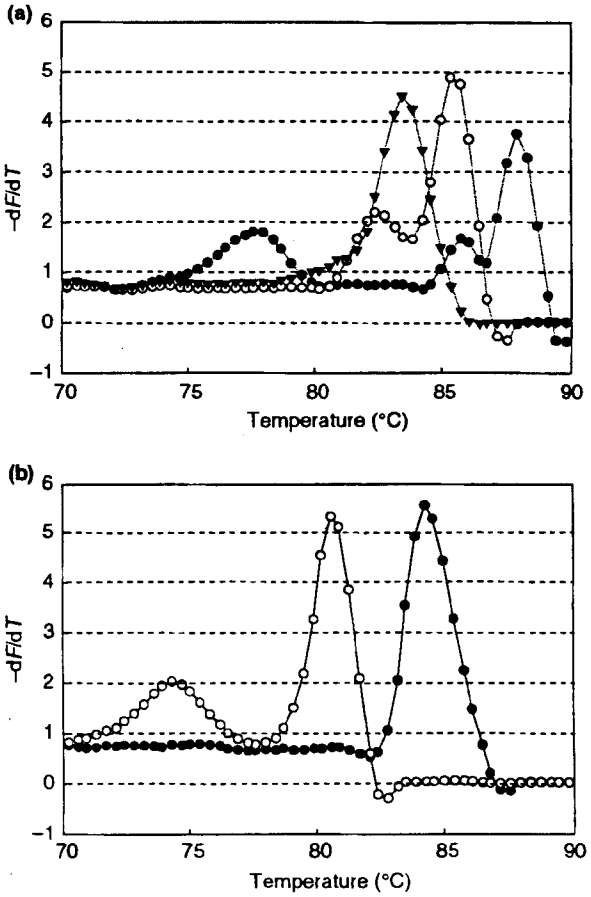

Fig. 1 Results of specificity testing using the iroB, ST, Fim, Sal, and INVA primer sets with *Salm. enterica* Schwarzengrund at a concentration of 6×10^4 CFU rxn^{-1}. Primer sets: (a) iroB (●), ST (○), Fim (▼); (b) Sal (●), INVA (○)

The Fim primer set produced a single 85 bp product and a single peak in the dissociation plot. However, primer dimers formed in the negative control reaction of the same size and position on the dissociation plot as the expected product. Testing this primer set at a reduced concentration of 250 nmol l^{-1} could help to reduce primer dimer formation. However, even if the size of the primer dimer seen in the negative control reaction was reduced, it may be difficult to interpret results from the *Salm. enterica* reactions because it will not be possible to tell if the fluorescent signal generated is from the target product, a primer dimer, or a combination of both. As it was not possible to distinguish between the *Salm. enterica* reactions and the negative control reactions in either dissociation analysis or gel electrophoresis, the Fim primer set was not used for further experimentation.

The INVA primer set results indicated that products of the correct size were formed with all *Salm. enterica* serovars tested. Figure 1(b) shows a large primary peak at 80°C along with a smaller secondary peak (which may have been a primer dimer) at a melting temperature of about 74°C. The larger peak in the dissociation analysis corresponded to the 244 bp product seen in the agarose gel (data not shown), as expected. As the primary product peak was clearly distinguishable from the smaller peak, the INVA primer set was used for sensitivity testing.

All *Salm. enterica* serovars tested with Sal were detected at 10^3 CFU rxn^{-1} with little to no primer dimer formation. The peak seen at 85°C in the dissociation plot of Fig. 1(b) corresponded to the expected 275 bp product seen in the gel analysis. The Sal primer set yielded the expected product for all *Salm. enterica* serovars and was therefore selected for sensitivity analysis.

Sensitivity of the Sal and INVA primer sets

In the case of both Sal and INVA primer sets, the dissociation plots showed that as the amount of initial template decreased, the height of the primer dimer peak increased and the height of the product peak decreased (data not shown). The detection limits of the Sal and INVA primer sets were approx. 3×10^3 and 3×10^4 CFU rxn^{-1}, respectively.

Primer analysis and design

As a lower detection limit was desired, software was used to compare the Sal and INVA primer sets with a new primer set design. Analysis of INVA and Sal indicated that these primer sets might form primer dimers, as indicated by the relatively large negative ΔG values associated with different primer dimer formations (data not shown).

A new primer set, Sen (*Salm. enterica*), also targeting the *invA* gene was designed and tested for *Salm. enterica* specificity using *Salm. enterica* and non-*Salmonella* isolates. The detection limit of Sen was also tested with *Salm. enterica* Newport. Table 2 gives the Sen primer set sequence. The software results indicated that only the reverse primer, Sen-2, has the potential for false priming on the *invA* gene (data not shown).

Sen primer set specificity and sensitivity

All *Salmonella* serovars shown in Table 1 formed a single product near the expected size, based on the real-time PCR dissociation plots and agarose gel electrophoresis results (data not shown). Sen primer set PCR products were sequenced in both directions and the product formed was the expected length of 172 bp. A BLAST analysis of the sequence results indicated that the correct sequence of bases was formed (accession number: AE008832, McClelland *et al.* 2001). None of the non-*Salmonella* bacteria crossed the real-time PCR threshold value, Rn = 0·5, over the course of 40 cycles (data not shown). Therefore, this primer set was considered specific for *Salm. enterica*.

Typical sensitivity results with the Sen primer set are shown in Fig. 2. Figure 2(a) shows that *Salm. enterica* Newport was detectable from 6×10^5 to 6×10^0 CFU rxn^{-1}, while the negative control reaction did not generate visible dsDNA. Additionally, as seen in Fig. 2b, no primer dimers were formed with the *Salm. enterica* Newport dilutions and all product peaks were found at a temperature of 82 ± 1°C.

The Sen sensitivity results indicate that the negative control reactions did not generate enough dsDNA to cross the relative fluorescence threshold value. As no negative control C_T values were available for comparison with *Salm. enterica* reaction C_T values, no statistical tests were performed. Detection limits of 80, 6 and 20 CFU rxn^{-1} were found in three separate runs, giving an average detection limit of 35 CFU rxn^{-1} with a standard deviation of 39 CFU rxn^{-1}.

DISCUSSION

In comparing the six primer sets for detection of *Salm. enterica* with real-time PCR, we chose to use pure cultures in sterile water as the first step in assay development. The formation of primer dimers in real-time PCR applications generates false-positive signals (Higuchi *et al.* 1992). These primer dimers ultimately lead to poor detection limits even without the presence of inhibiting substances found in field samples.

With varying degrees of amplification, all primer sets tested with the *Salm. enterica* serovars shown in Table 1 formed PCR products of the expected sizes at

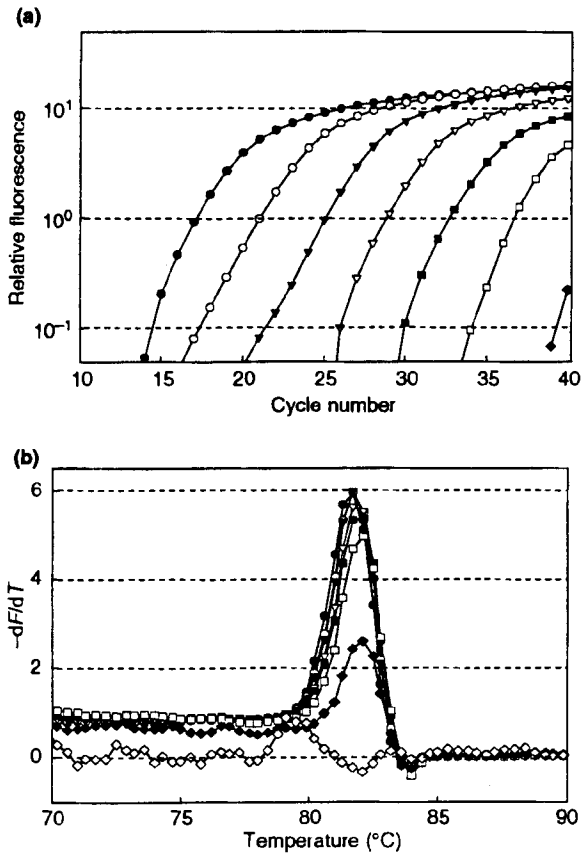

Fig. 2 Amplification and dissociation plots with the Sen primer set amplifying the DNA of *Salm. enterica* Newport. Cell concentrations: (a) 6×10^5 CFU rxn^{-1} (●), 6×10^4 CFU rxn^{-1} (○), 6×10^3 CFU rxn^{-1} (▼), 6×10^2 CFU rxn^{-1} (▽), 6×10^1 CFU rxn^{-1} (■), 6×10^0 CFU rxn^{-1} (□), 6×10^{-1} CFU rxn^{-1} (◆). Cell concentrations: (b) 6×10^5 CFU rxn^{-1} (●), 6×10^4 CFU rxn^{-1} (○), 6×10^3 CFU rxn^{-1} (▼), 6×10^2 CFU rxn^{-1} (▽), 6×10^1 CFU rxn^{-1} (■), 6×10^0 CFU rxn^{-1} (□), 6×10^{-1} CFU rxn^{-1} (◆), negative control (◇)

10^3 CFU rxn^{-1}. However, multiple peaks were seen in the dissociation plots of the ST and iroB primer sets with most of the *Salm. enterica* serovars tested, and this may have been the result of two products formed that were close to one another or a single product with a DNA sequence that generates a complex melting profile. Li *et al.* (2003) demonstrated that complex melting profiles can result from small single PCR products with SYBR Green dye. Their data indicate that regions of high A/T and G/C bases contribute to the shape of the melting profile of a product. A complex melting profile in the dissociation analysis of a PCR product that shows a single band on a gel requires sequencing to confirm that one product was actually formed and co-migrating bands were not present.

With the exception of one sample at 8 CFU rxn^{-1}, all dissociation plots for Sen primer products displayed single peaks larger than the background signal. The dissociation analyses confirmed that the correct melting temperature was found in each of the Sen primer set products generated.

Although extraction and enrichment procedures may improve the sensitivity of PCR, both steps are time consuming and labour intensive and, thus, were not used here to prepare the samples. Without an enrichment technique to increase levels of target DNA or an extraction process to purify DNA from a sample, the choice of primer set is critical in a real-time PCR application. At high template concentrations (10^6–10^5 CFU rxn^{-1}), both the Sal and INVA primer sets were consistently able to amplify the target fragment of interest. However, PCR with less template often generated primer dimers, ultimately yielding poor detection limits. The primer software was valuable in predicting the tendency for dimer formation and designing the Sen primer set which was ultimately the most sensitive primer set we tested. Using primers developed for traditional PCR may lower real-time assay sensitivity. Therefore, new primers are required specifically for real-time PCR.

The ultimate goal of this research is to build an automated biosensor for field use to screen for pathogens in the irrigation/wash water from fruits and vegetables. Jackson *et al.* (2003) started this work and developed components for such a biosensor. An ideal biosensor rapidly confirms the presence or absence of its target. While enrichment techniques have been used prior to PCR for sensitive detection of *Salmonella* (Eyigor *et al.* 2002; Bhagwat 2004), these procedures typically take hours and such practices are incompatible with the goal of rapid detection. In order to improve our detection limit, an automated concentration mechanism is under development for the purpose of increasing the cell densities of liquid test samples. DNA extraction steps may also improve PCR target detection, but not all steps in an extraction process are easily automated. Obtaining a specific and sensitive primer set for real-time PCR detection of *Salm. enterica* under ideal conditions was the first step in the development of an automated biosensor. The next step will be testing *Salm. enterica* detection performance in spiked water samples from produce operations.

ACKNOWLEDGEMENTS

We thank Sharon Abbott, Microbial Diseases Laboratory, California Department of Health Services, Berkeley and Nicole Perna, University of Wisconsin-Madison, for providing the strains. This research was funded in part by the International Life Sciences Institute and the United States Department of Agriculture, Agricultural Research Service CRIS project 5325-42000-040.

REFERENCES

Barak, J.D., Whitehand, L.C. and Charkowski, A.O. (2002) Differences in attachment of *Salm. enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. *Applied and Environmental Microbiology* 68, 4758–4763.

Barak, J.D., Chue, B. and Mills, D. (2003) Recovery of surface bacteria from and surface sanitization of cantaloupes. *Journal of Food Protection* 66, 1805–1810.

Bäumler, A.J., Heffron, F. and Reissbrodt, R. (1997) Rapid detection of *Salm. enterica* with primers specific for *iroB*. *Journal of Clinical Microbiology* 35, 1224–1230.

Bhagwat, A.A. (2004) Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. *Food Microbiology*, 21, 73–78.

Chiu, H.-C. and Ou, J.T. (1996) Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *Journal of Clinical Microbiology* 34, 2619–2622.

Cohen, H.J., Mechanda, S.M. and Lin, W. (1996) PCR amplification of the *fimA* gene sequence of *Salmonella* Typhimurium, a specific method for detection of *Salmonella* spp. *Applied and Environmental Microbiology* 62, 4303–4308.

Eyigor, A., Carli, K.T. and Unal, C.B. (2002) Implementation of real-time PCR to tetrathionate broth enrichment step of *Salmonella* detection in poultry. *Letters in Applied Microbiology* 34, 37–41.

Gooding, C.M. and Choudary, P.V. (1999) Comparison of different primers for rapid detection of *Salmonella* using the polymerase chain reaction. *Molecular and Cellular Probes* 13, 341–347.

Higuchi, R., Dollinger, G., Walsh, S.P. and Griffith, R. (1992) Simultaneous amplification and detection of specific DNA sequences. *BioTechnology* 10, 413–417.

Hoorfar, J. Ahrens, P. and Rådström, P. (2000) Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. *Journal of Clinical Microbiology* 38, 3429–3435.

Inami, G.B. and Moler, S.E. (1999) Detection and isolation of *Salmonella* from naturally contaminated alfalfa seeds following an outbreak investigation. *Journal of Food Protection* 62, 662–664.

Jackson, E., Delwiche, M., Barak, J., Charkowski, A. and Suslow, T. (2003) Sensor components for PCR detection of *Salmonella* in alfalfa sprout irrigation water. *Transactions of the ASAE* (in press).

Li, W., Xi, B., Yang, W., Hawkins, M. and Schubart, U.K. (2003) Complex DNA melting profiles of small PCR products revealed using SYBR Green I. *BioTechniques* 35, 702–706.

Malorny, B., Hoofar, J., Bunge, C. and Helmuth, R. (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and Environmental Microbiology* 69, 290–296.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J. *et al.* (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* 413, 852–856.

Mohle-Boetani, J.C., Reporter, R., Werner, S.B., Abbott, S., Farrar, J., Waterman, S.H. and Vugia, D.J. (1999) An outbreak of *Salmonella* serogroup Saphra due to cantaloupes from Mexico. *Journal of Infectious Diseases* 180, 1361–1364.

Mohle-Boetani, J.C., Farrar, J.A., Werner, S.B., Minassian, D., Bryant, R., Abbott, S., Slutsker, L. and Vugia, D.J. (2001) *Escherichia coli* O157 and *Salmonella* infections associated with sprouts in California, 1996–1998. *Annals of Internal Medicine* 135, 239–247.

Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G. and Colin, P. (1999) Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella* Enteritidis and *Salmonella* Typhimurium from environmental swabs of poultry houses. *Letters in Applied Microbiology* 28, 113–117.

Wachtel, M.R., Whitehand, L.C. and Mandrell, R.E. (2002) Prevalence of *Escherichia coli* associated with a cabbage crop inadvertently irrigated with partially treated sewage wastewater. *Journal of Food Protection* 65, 471–475.

Wang, R.-F., Cao, W.-W. and Cerniglia, C.E. (1997) A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *Journal of Applied Microbiology* 83, 727–736.

Ziemer, C.J. and Steadham, S.R. (2003) Evaluation of the specificity of *Salmonella* PCR primers using various intestinal bacterial species. *Letters in Applied Microbiology* 37, 463–469.

CLINICAL MICROBIOLOGY REVIEWS, Oct. 2000, p. 559–570 Vol. 13, No. 4
0893-8512/00/$04.00+0

Multiplex PCR: Optimization and Application in Diagnostic Virology

ELFATH M. ELNIFRO,[1] AHMED M. ASHSHI,[1] ROBERT J. COOPER,[1] AND PAUL E. KLAPPER[1,2]*

School of Medicine, The University of Manchester,[1] and Clinical Virology, Central Manchester Healthcare Trust,[2] Manchester, United Kingdom

INTRODUCTION

During the past decade, advances in PCR technology and other DNA signal and target amplification techniques have resulted in these molecular diagnostics becoming key procedures (4, 107, 117). Such techniques are conceptually simple, highly specific, sensitive, and amenable to full automation (54, 115). The most mature of these technologies, PCR, is in one variant or another now common in research laboratories and is used increasingly in routine diagnostic laboratory settings and undergraduate and high-school teaching (32, 38, 40, 101). In diagnostic laboratories the use of PCR is limited by cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, a variant termed multiplex PCR has been described. In multiplex PCR more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including gene deletion analysis (19, 20), mutation and polymorphism analysis (86, 96), quantitative analysis (94, 124), and RNA detection (51, 126). In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi, and/or parasites. A representative list of such agents is shown in Table 1.

Based upon our own experience with multiplex PCR and those of other authors appearing in the literature during the last 10 years, we review the theoretical and practical basis of the development and optimization of multiplex PCR systems and discuss the application and potential of this technique in the field of diagnostic virology.

* Corresponding author. Mailing address: Clinical Virology, 3rd Floor, Clinical Sciences Building, Manchester Royal Infirmary, Oxford Rd., Manchester M13 9WL, United Kingdom. Phone: 44 161 276 8849. Fax: 44 161 276 8840. E-mail: pklapper@mri5.cmht.nwest.nhs.uk.

PRINCIPLE AND DEVELOPMENT OF MULTIPLEX PCR

A number of review and research articles have provided detailed descriptions of the key parameters that may influence the performance of standard (uniplex) PCR (17, 57, 88, 91, 112). Fewer publications discuss multiplex PCR (18, 28, 43).

Primers and Multiplex PCR Efficiency

The first few rounds of thermal cycling have substantial effect on the overall sensitivity and specificity of PCR (92). Assuming efficient denaturation of the target, overall success of specific amplification depends on the rate at which primers anneal to their target and the rate at which annealed primers are extended along the desired sequence during the early, middle, and late cycles of the amplification. Factors preventing optimal annealing rates include poorly designed primers and suboptimal buffer constituents and annealing temperature. The extension rate of specific primer-target hybrids depends on the activity of the enzyme, availability of essential components such as deoxyribonucleoside triphosphates (dNTPs), and the nature of the target DNA. Thus, the majority of modifications to improve PCR performance have been directed towards the factors affecting annealing and/or extension rates.

The optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets (76). The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers (9). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or

TABLE 1. Representative list of applications of multiplex PCR to the diagnosis of infectious diseases

Infectious agent	Pathogens targeted	Clinical manifestation(s) and/or specimen	Reference(s)
Virus	HIV-1, HIV-2, HTLV-1, and HTLV-2	Blood	45
	HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EVs[a]	Meningitis, encephalitis, or meningoencephalitis; CSF	13, 14
Bacterium	*Haemophilus influenzae*, *Streptococcus pneumonia*, *Mycoplasma catarrhalis*, and *Alloiococcus otitidis*	Upper respiratory tract	42
	Campylobacter jejuni and *Campylobacter coli*	Human campylobacteriosis	37
	Actinomyces actinomycetemcomitans, *Porphyromonas intermedia*, and *Porphyromonas gingivalis*	Periodontal infection	34
	N. gonorrhoeae and *C. trachomatis*	Genital infections	60, 118
	C. trachomatis, *N. gonorrhoeae*, *Ureaplasma urealyticum*, and *M. genitalium*	Genital infections	59
Parasite	*Giardia lamblia* and *Cryptosporidium parvum*	Diarrheal disease; water	52, 89
	Leishmania spp.	Leishmaniasis	5, 39
Combination	HSV, *H. ducreyi*, and *T. pallidum*	Genital ulcer disease	7, 73
	HPVs, HSV, and *C. trachomatis*	Genital swabs	64
	Adenovirus, HSV, and *C. trachomatis*	Keratoconjunctivitis	Yeo et al.[b]
	EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *M. pneumoniae*, and *C. pneumoniae*	Acute respiratory tract infections	36

[a] EVs, enteroviruses.
[b] Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997.

reduce such nonspecific interactions. Empirical testing and a trial-and-error approach may have to be used when testing several primer pairs, because there are no means to predict the performance characteristics of a selected primer pair even among those that satisfy the general parameters of primer design (43). However, special attention to primer design parameters such as homology of primers with their target nucleic acid sequences, their length, the GC content, and their concentration have to be considered (26, 65, 72, 88, 95, 120). Ideally, all the primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures (primer length of 18 to 30 bp or more and a GC content of 35 to 60% may prove satisfactory) and should not display significant homology either internally or to one another (17, 26, 43).

Preferential amplification of one target sequence over another (bias in template-to-product ratios) is a known phenomenon in multiplex PCRs that are designed to amplify more than one target simultaneously (68, 76, 109). Based on both theoretical modeling and experimental studies, two major classes of processes that induce this bias have been identified, PCR drift and PCR selection (108). PCR drift is a bias assumed to be due to stochastic fluctuation in the interactions of PCR reagents particularly in the early cycles, which could arise in the presence of very low template concentrations (26, 68); variations in the thermal profiles of a thermocycler, resulting in unequal ramping temperatures; or simple experimental error. PCR selection, on the other hand, is defined as a mechanism which inherently favors the amplification of certain templates due to the properties of the target, the target's flanking sequences, or the entire target genome. These properties include interregion differences in GC content, leading to preferential denaturation; higher binding efficiency because of GC-rich primers; differential accessibility of targets within genomes due to secondary structures; and the gene copy number within a genome. In addition, the choice of primers has been shown to be crucial to avoid PCR selection. Amplification biases that were strongly dependent on the choice of primers and dependent to a lesser extent on the templates have been described (100). Some primer pairs with high amplification efficiency resulted in templates being saturated (plateau phase), while other primer pairs produced product independent of starting template concentrations. Primers with lower amplification efficiency resulted in product concentrations below the saturation concentrations, and depending on the template, either the expected product ratio or bias was observed.

Other PCR Components

Alteration of other PCR components such as PCR buffer constituents, dNTPs, and enzyme concentrations in multiplex PCR over those reported for most uniplex PCRs usually results in little, if any, improvement in the sensitivity or specificity of the test. Increasing the concentration of these factors may increase the likelihood of mis-priming with subsequent production of spurious nonspecific amplification products. However, optimization of these components in multiplex PCRs that are designed for simultaneous amplification of multiple targets may prove beneficial. For example, in the multiplex PCR for the dystrophin gene (nine genomic targets), a *Taq* DNA polymerase concentration (with an appropriate increase in $MgCl_2$ concentration) four to five times greater than that required in uniplex PCR was necessary to achieve optimal nucleic acid amplification (19). Variation in concentrations of reaction components above those used in uniplex PCR probably reflects the competitive nature of the PCR process. The desired target DNA can be outcompeted by the more efficient amplification of other targets (including nonspecific products), leading to decreases in the efficiency of the amplification of the desired targets and hence sensitivity of the reaction (79).

PCR additives, such as dimethyl sulfoxide, glycerol, bovine serum albumin, or betaine, have been reported to be of benefit in multiplex PCRs (49, 62). The components may act to prevent the stalling of DNA polymerization, which can occur through the formation of secondary structures within regions of template DNA during the extension process (44). Such cosolvents may also act as destabilizing agents, reducing the melting temperature of GC-rich sequences, or as osmopro-

tectants, increasing the resistance of the polymerase to denaturation (44, 83).

Variations in Methodology To Improve Sensitivity and Specificity

A straightforward solution to difficulties encountered in the development of multiplex PCR has been the use of hot start PCR (21) and/or nested PCR (123). The former often eliminates nonspecific reactions (particularly production of primer dimers) caused by primer annealing at low temperature (4 to 25°C) before commencement of thermocycling (21). The procedure has recently been made more practicable through the use of a nonmechanical hot start methodology which involves the use of a form of *Taq* polymerase, for example, Amplitaq Gold (Roche Diagnostics), which is activated only if the reaction mixture is heated at approximately 94°C for 10 min (the first denaturation step) (8, 53). Nested PCR increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets. Although this adaptation is undoubtedly effective in most cases, it also considerably complicates the practical application of PCR. The second round of amplification delays results, increases the possibility of cross-contamination, and may complicate automation.

General Considerations for Multiplex PCR Development

Development of multiplex PCRs should follow a rational approach for the inclusion or exclusion of specific pathogens in the assay. These pathogens can be organ system specific or symptom specific with respect to the age of the patient and the epidemiological characteristics of these pathogens. PCR conditions, such as compatibility among the primers within the reaction mixture such that there is no interference, is of great technical importance. The primer pairs must be inclusive for as many strains of the target pathogen as possible, and depending on the amplicon detection method, their targets are easily resolvable. The latter may be achieved by using primer pairs that result in PCR products that can be separated and clearly visualized using gel electrophoresis or hybridization probes with maximum specificity. Prior to application in a clinical setting, multiplex PCRs must be evaluated for their sensitivity as compared with their corresponding uniplex PCRs using both serial dilutions of the target DNA and clinical specimens.

Wherever possible, multiplex PCRs should avoid the use of nested primers requiring a second round of amplification. The latter is a major contributor to false-positive results due to carryover contamination, although anticontamination protocols including PCR controls (reaction and specimens extraction controls) must be implemented in all PCR-based protocols (55). Likewise, precautions and methodologies to avoid false-negative results due to reaction failure have to be considered (104). Multiplex PCRs that amplify target sequences along with the presence of external or internal control target nucleic acids to indicate reaction failure have been developed (49, 62, 69).

APPLICATION OF MULTIPLEX PCR IN DIAGNOSTIC VIROLOGY

During the last decade, a number of studies have demonstrated the practicality of identifying viral pathogens in many clinical and epidemiological settings using multiplex PCR (Table 2). The technique has been used to screen for individual or symptom-associated viruses and examine associations of virus infection with disease. In addition, the technique has been shown to be a powerful and cost-effective tool for typing and subtyping virus strains in different epidemiological studies.

Neurotropic Viruses

PCR has proved to be a powerful tool for investigating meningitis and encephalitis caused by a variety of viruses. In neurological disease the requirement of rapid and reliable diagnosis to provide a rational basis for chemotherapy and limit unnecessary procedures and irrelevant therapy has driven development. The wide range of viruses associated with neurological disease includes herpes simplex virus (HSV); cytomegalovirus (CMV); varicella-zoster virus (VZV); Epstein-Barr virus (EBV); human herpes virus 6 (HHV-6); the enterovirus group, including echoviruses, polioviruses, and coxsackieviruses; adenoviruses; JC and BK viruses; arenaviruses; paramyxoviruses; rabies; and arboviruses. In view of the large number of potentially neuroinvasive viruses and because of the limited volume of the most useful diagnostic specimen—cerebrospinal fluid (CSF)—a number of multiplex PCRs have been developed (12–15, 82, 102).

The feasibility of simultaneous screening for viruses, bacteria, and parasites in CSF specimens from patients with aseptic meningitis or encephalitis has been described (82). This study by Read et al. (82) utilized three nested multiplex PCRs for detection of HSV and VZV; EBV and HHV-6; and members of the enterovirus group and echovirus type 22 and 23. In addition, two uniplex PCRs were used for detection of CMV and JC virus. In a total of 2,233 CSF specimens from 2,162 patients, the PCR was positive in 147 specimens from 143 patients (6.6% of all patients) including enteroviruses (77 patients), HSV-1 (20 patients), VZV (7 patients), HSV type 2 (HSV-2) (6 patients), CMV (3 patients), JC virus (2 patients), and HHV-6 (1 patient). All PCR assays remained negative with 28 control CSF specimens. The clinical sensitivity and specificity of this PCR were not determined because full clinical information was not available for all of the patients.

A nested multiplex PCR for detection and differentiation of HSV-1 and -2 on the basis of PCR product size has also been described (14). In a prospective analysis, a total of 417 CSF specimens obtained from 395 consecutive patients with clinical suspicion of HSV encephalitis, meningitis, or meningoencephalitis were tested by multiplex PCR. The test was positive for HSV-1 in 11 specimens (2.6%) from 10 patients and for HSV-2 in 4 specimens (1.0%) from 3 patients; no coinfection with both types was reported. The same multiplex PCR was used to test a total of 178 CSF samples obtained from 171 patients with clinical suspicion of herpes virus infection (15). The assay was positive for HSV-1 in three samples (1.7%) from two patients (1.2%) and for HSV-2 in one sample, and one patient tested positive in a nested uniplex CMV PCR. A similar procedure to detect the DNA of both viruses (HSV-1 and -2) was applied to CSF samples from 918 human immunodeficiency virus (HIV)-infected patients with neurological symptoms (22). In patients for whom a diagnosis was confirmed at autopsy, the test was positive for HSV-1 or -2 for 19 patients (2%), producing a sensitivity and specificity of 100 and 99.6%, respectively.

The first nested multiplex PCR for detection and typing of herpesviruses (HSV-1 and -2, VZV, CMV, HHV-6, and EBV) was applied to CSF from patients with meningitis, encephalitis, and other clinical syndromes (102). By utilizing equimolar concentrations of primers aligning the 3′ ends with one of two consensus regions within the herpesvirus DNA polymerase gene and the 5′ ends with the related or nonrelated sequences of each agent to be amplified, the first round of amplification yielded a 194-bp fragment indicating the presence of herpes-

TABLE 2. Application of multiplex PCR for diagnosis of viral infections

Clinical manifestation(s)	Specimen(s)	Viruses and/or other agent(s) targeted	Reference(s)
Meningitis, encephalitis, and/or meningo-encephalitis	CSF	HSV-1, HSV-2, and CMV	14
		HSV and VZV; EBV and HHV-6	81
		HSV-1, HSV-2, VZV, CMV, HHV-6, and EBV	102
		HSV-1 and HSV-2	15
		HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EVs[a]	12, 13
		CMV, EBV, HHV-6, HHV-7, and HHV-8	77
		EBV and *T. gondii*	87
Upper and lower respiratory infections	Throat, nose, and nasopharyngeal swabs; nasopharyngeal and endotracheal aspirates; bronchoalveolar lavage	Influenza viruses A and B	30
		PIV types 1, 2, and 3	27
		Influenza virus and RSV	98
		RSVs A and B, influenza viruses A and B, PIV types 1, 2, and 3	33
		RSV, PIVs, adenovirus	74
		EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *M. pneumoniae*, *C. pneumoniae*	36
Conjunctivitis, keratitis, keratoconjunctivitis	Conjunctival and corneal swabs	HSV-1 and HSV-2	15
		Adenovirus and HSV	49
		Adenovirus, HSV, and *C. trachomatis*	Yeo et al.[6]
Genital ulcer disease	Genital ulcer swabs	HSV, *H. ducreyi*, and *T. pallidum*	7, 66, 73
Genital lesions	Lesion and endocervical swabs	HPVs, HSV, and *C. trachomatis*	64
HPV-associated genital disease	Cervical scrapings, smears, and biopsies; vaginal and vulval swabs	HPVs	23, 35, 56, 75, 97, 123
Vesicular rashes	Vesicle fluids	HSV and VZV	3
Hepatitis	Serum and plasma samples	HBV genotypes	84
		HCV, HGV, and GB viruses	16
Immunocompromised status	Plasma	HHV-6 and HHV-7	67
	Blood	HIV-1, HIV-2, HTLV-1, HTLV-2	45

[a] EVs, enteroviruses.
[b] Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997.

virus. The second round of amplification utilizing primer mixtures contained nonhomologous and type-specific primers selected from different regions of the aligned DNA polymerase genes of human herpesviruses produce a product with a different size for each related virus. The method amplified the corresponding virus in infected cells and in five clinical samples (HSV-1 PCR-positive CSF from a patient with encephalitis, HSV-2 PCR-positive CSF from a patient with meningitis, VZV culture-positive vesicular fluid from a patient with shingles, CMV culture-positive urine from a congenitally infected patient, and EBV PCR-positive peripheral blood from a patient with a lymphoproliferative syndrome). In addition, the use of primers targeting consensus regions may allow recognition of new, undescribed human herpesviruses. The detection of a 194-bp fragment after the first reaction with no positive signal in the second round of amplification could reflect the detection of a new human herpesvirus. The test was further modified to include a reverse transcription step and primer pairs to detect enterovirus cDNA (12). This PCR was then evaluated in 21 patients with etiologically well-characterized aseptic meningitis and encephalitis. HSV DNA was detected in nine patients, VZV DNA was detected in 6 patients, and enterovirus RNA was detected in 6 patients. The test was further evaluated for detection of these same viruses in CSF samples by a prospective study of 200 neurological-disease patients suspected to have viral infections. Enterovirus was detected in 49 patients, HSV was detected in 3 patients, VZV was detected in 6 patients, CMV was detected in 12 patients, EBV was detected in 2 patients, CMV and HSV were detected in one AIDS patient with encephalitis, and CMV and EBV were detected in another AIDS patient with polyradiculomyelitis. For detection of echovirus 30 in 50 patients with aseptic meningitis, the multiplex reverse transcription (RT)-PCR was more sensitive (90% sensitivity) than cell culture (26% sensitivity) and the Amplicor EV test (86% sensitivity). These studies demonstrate the utility of this multiplex RT-PCR for detection of enteroviruses and herpesviruses in CSF samples from patients with various neurological manifestations and the usefulness of the technique in patient management and design of antiviral therapy.

In the United Kingdom, a nested multiplex PCR for the detection of HSV-1 and -2, VZV, and enteroviruses, the four most common causes of viral meningitis and encephalitis, was developed and evaluated using a total of 1,683 consecutive CSF samples (81). The test was positive in 138 (8.2%) of the specimens (enteroviruses in 51 samples, HSV-2 in 33 samples, VZV in 28 samples, and HSV-1 in 25 samples). Of the 51 patients positive for enterovirus RNA, 17 were babies less than 6 months old in whom the CNS infection was detected as part

of a general infection screen and 34 patients were older children and adults who had encephalitis and meningitis. In the group positive for VZV (28 patients), 16 patients had meningitis and 10 had encephalitis but clinical details were not available for 2 patients. HSV-1 was detected in two babies less than 6 months old and in 23 adults (22 had encephalitis and 1 had a benign lymphocytic meningitis). The HSV-2-positive patients (33 patients) included five babies less than 6 months old, two adults with meningoencephalitis, and 26 patients more than 6 months old with benign lymphocytic meningitis. These tests proved suitable for routine use in a diagnostic laboratory and highlighted the importance of screening for more than one virus in patients with meningitis and encephalitis.

Although the studies described above (13–15, 81, 82, 102) produced satisfactory results in terms of simultaneous screening for neurological manifestation-associated viruses (for example herpesviruses), the multiplex PCRs developed utilized a nested strategy. The latter as described earlier may increase the chance of false-positive results due to contamination and may also complicate automation. A recent study (63) utilized a PCR assay which precludes the use of nested primers for simultaneous amplification of herpesviruses DNAs. This assay, termed consensus PCR, uses a pair of "stair" primers, which are based on consensus sequences selected from within the DNA polymerase gene and were 76 to 86% identical to the genomic sequences of the six herpesviruses (HSV-1, HSV-2, CMV, EBV, VZV, and HHV-6) that may infect the CNS. Each stair primer used comprised an equimolar mixture of 11 oligonucleotides corresponding to a consensus sequence: all primers had the same 5′ end but extended for 20 to 30 nucleotides in the 3′ direction. The PCR products were analyzed by hybridization in microtiter plates using virus-specific, biotinylated oligonucleotide probes. The consensus PCR was evaluated using 142 CSF samples previously tested by standard uniplex PCRs. Eighteen samples (12.7%) tested positive by the uniplex PCRs, and 37 (26%) tested positive by the consensus PCR, including 3 samples that had coinfections (CMV, VZV, and HSV-2; VZV and HSV-2; and CMV and HHV-6). Of the 142 CSF samples, 103 were classified as negative by both the uniplex PCRs and the consensus PCR. In addition, the test showed high diagnostic utility in that several cases were found to be positive for viruses for which tests were not requested by the clinician.

The problem in evaluating all of the aforementioned multiplex PCR studies is that most have not included complete patient detail. Thus, while many positive results have been related to compatible clinical illness, positive results are not reported in all patients with similar conditions. Nevertheless, the multiplex PCR detects more positive specimens and is more rapid than conventional techniques such as culture or serology. However, the latter procedures are either insensitive or slow and make an unsatisfactory yardstick ("gold standard") against which to measure the accuracy of multiplex PCR. Because uniplex PCR has more data available and thus is better substantiated as to its clinical value, results of multiplex PCR should be compared with those of uniplex PCR to ensure that multiplex PCR has equivalent sensitivity, specificity, and clinical relevance.

Respiratory Viruses

Viruses that commonly cause respiratory infection include respiratory syncytial virus (RSV), influenza viruses and parainfluenza viruses (PIV), and adenovirus, especially in infants and young children. Infection with these viruses may result in severe lower or upper respiratory tract disease requiring hospitalization. Thus, sensitive and rapid testing for these viruses is crucial to reduce the potential of nosocomial transmission to high-risk patients, limit unnecessary antibiotic use, and direct appropriate therapy following a specific diagnosis (119). For this reason, a number of studies have aimed to develop and evaluate multiplex PCR for detection of these viruses and provided substantial evidence of the utility of this technique as an important tool for management of patients presenting with respiratory infections. A number of studies have utilized multiplex PCR to both detect and type or subtype influenza viruses, PIVs, and RSV in clinical specimens and are summarized below.

A nested multiplex RT-PCR which included three primer pairs in each round of amplification was utilized for the simultaneous detection, typing, and subtyping of influenza type A (H3N2 and H1N1) and type B viruses in a prospective surveillance of influenza in England in the 1995–1996 winter season (30). A total of 619 combined nose and throat swabs from patients with an influenza-like illness were analyzed by culture and multiplex PCR. The multiplex RT-PCR detected influenza viruses in 246 (39.7%) samples compared to the 200 (32.3%) which yielded influenza viruses in culture. In addition, there was excellent correlation between the multiplex RT-PCR and culture for typing and subtyping of influenza viruses (100%) and for temporal detection of influenza A H3N2 and H1N1 viruses. It was concluded that whereas the multiplex RT-PCR demonstrated its utility in detection of influenza viruses in patients with influenza-like illness, patients with influenza-like illness who are negative for influenza viruses may harbor a pathogen(s) producing a syndrome difficult to distinguish clinically from true influenza (for example, RSV). Indeed, when this multiplex RT-PCR was modified so that it was capable of detecting and subtyping influenza A (H1N1 and H3N2) and B viruses as well as RSV subtypes A and B in respiratory clinical samples (98), the assay again demonstrated excellent (100%) correlation with the results of culture and serology. The ability of the test to detect viral coinfection in both simulated specimens and clinical samples was also demonstrated.

A nested multiplex RT-PCR using three primer pairs was developed to detect PIV types 1, 2, and 3 in throat and nasopharyngeal swabs (27). In the first round of amplification, similar-size fragments are produced. In the second round of amplification a series of three internal primer pairs are introduced, producing type-specific amplicons that were easily differentiated based on size upon gel electrophoresis. The test detected and correctly typed PIV in 15 isolates and 26 of 30 (87%) previously positive nasopharyngeal specimens but remained negative in naso- or oropharyngeal specimens and/or culture isolates of 33 unrelated respiratory tract pathogens. In a modified version, the test was also used to detect RSV and adenovirus utilizing five primer sets to amplify cDNA of RSV subtypes A and B; PIV types 1, 2, and 3; and DNA of adenovirus types 1 to 7 (74). The test was sensitive and specific for all 12 tissue culture-grown prototype viruses and when applied to respiratory specimens was more sensitive (41 of 112) than direct immunofluorescence or antigen detection following culture (34 of 112). Among positive samples, multiple respiratory viruses were found in four specimens, further illustrating the potential utility of this multiplex PCR assay.

A multiplex quantitative RT-PCR enzyme hybridization assay (Hexaplex; Prodesse, Inc., Milwaukee, Wis.) which combines primers originating from highly conserved regions of 7 respiratory viruses (RSV subtypes A and B; PIV types 1, 2, and 3; and influenza viruses A and B) with probes for the detection of PCR products using enzyme hybridization assay has also been described (33). The assay provides rapid simultaneous

detection, identification, and quantitation of these viruses in nasal wash specimens in a single test. The primer and the probes utilized were evaluated using multiple virus isolates from each group and resulted in specific PCR products from all tissue culture-positive specimens without cross-reactivity among these seven viruses or with other common human respiratory viruses. The Hexaplex assay was applied on nasal wash specimens from 69 children with signs of lower respiratory tract infection and 40 specimens from asymptomatic children. Of the 69 specimens from symptomatic children, 37 were positive by the Hexaplex assay but only 29 of these were culture positive. Both the Hexaplex assay and virus culture were negative in the 40 nasal washes from the asymptomatic children. The Hexaplex assay was 100% sensitive and 98% specific in comparison with virus culture.

Multiplex PCR in combination with a heteroduplex mobility shift assay has proved to be a valuable and cost-effective tool for monitoring the emergence of new variants or new subtypes of influenza viruses arising through the phenomena of antigenic drift and antigenic shift (125, 126). On the basis of amplicon size, the amplification assay differentiates the variable region of the hemagglutinin genes of the H1 and H3 subtypes of influenza viruses A and B and the counterpart (hemagglutinin, esterase, and fusion gene) of influenza virus C. Variants within the same type or subtype are then identified by heteroduplex mobility shift assay of the amplicons. This approach proved to be a rapid, sensitive, and reliable method for the detection and typing of influenza virus and for screening for influenza virus variants, proving capable of identifying new influenza B virus variants (126).

More recently, it has been demonstrated that multiplex PCR is a useful and rapid diagnostic tool for the management of children with acute respiratory infections (36). This simplified hot start multiplex PCR allows simultaneous screening for nine different infectious agents (enterovirus, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*). The test was evaluated using clinical samples from 1,118 children with acute respiratory infections and was positive in 395 (35%) samples. Of these, 37.5% were positive for RSV, 20% were positive for influenza A virus, 12.9% were positive for adenovirus, 10.6% were positive for enterovirus, 8.1% were positive for *M. pneumoniae*, 4.3% were positive for PIV type 3, 3.5% were positive for PIV type 1, 2.8% were positive for influenza B virus, and 0.2% were positive for *Chlamydia trachomatis*. Seasonal variations in the rates of detection of the different organisms were noted. The test demonstrated levels of concordance of 95% for RSV and 98% for influenza A virus with the data obtained by commercially available enzyme immunoassay.

Genito-Urinary Infections

The utility of multiplex PCR in diagnosis of viruses associated with genital tract infection is reflected by the numerous reports detecting and typing human papilloma viruses (HPVs). These multiplex PCRs have a variety of formats but with a common aim of detecting and typing HPVs. These include PCRs which combined primers to produce a type-specific product size (97, 103) or those that utilized degenerate primers for HPV screening and strain-specific antisense primers for simple typing (56). In an alternative protocol, three consensus primer pairs simultaneously detected high-risk (for cervical carcinoma) HPV type 16 (HPV-16) and HPV-18 and low-risk HPV-6 and HPV-11, and primer pairs for more than 40 other HPV types were developed (consensus multiplex PCR) (124). This approach was considered to be a consensus multiplex PCR assay because all the primers are consensus and are multiplexed in the same PCR mixture for simultaneous amplification. One primer pair amplifies general HPV DNA from more than 40 types, including HPV-6, -11, -16, and -18 (450-bp PCR product), indicating HPV infection. Another primer pair generates a PCR product of 307 bp if HPV-16 and -18 DNA are present in the sample (high-risk HPV infection). The third consensus primer pair results in a PCR product of 550 bp with low-risk HPV infection (HPV-6 and -11). The assay was evaluated on exfoliated cell specimens obtained from 148 healthy women, biopsied specimens from 32 patients with condyloma acuminata, and biopsied specimens from 76 patients with invasive carcinoma of the uterine cervix. The multiplex PCR was positive in 47 (31.8%) of the samples from the 148 healthy women, but most of the positive samples were in samples from women infected with HPV types other than HPV-6 and -11 or HPV-16 and -18. All samples from patients with condyloma acuminata contained HPV DNA but mainly contained HPV-6/11 (87.5%), while high-risk HPV had a low prevalence rate (6.5%). HPVs were detected in samples from 69 (90.8%) of the 76 patients with cervical carcinoma, of which high-risk HPVs accounted for 82.9%. The method proved to be a simple, economical, and reliable tool for detection of HPV infection. The simultaneous amplification of the three HPV targets should allow rapid approach for distinguishing disease-related HPV types: low-risk HPVs (HPV-6 and HPV-11) involved in genital, benign lesions, such as warts or condylomas, and high-risk HPVs (HPV-16 and HPV-18), which are frequently found in malignant lesions of the lower genital tract. This should provide valuable information for monitoring and treating patients with HPV-related lesions, although the method lacks the capability to define individual HPV types, which limits its usefulness in epidemiological investigations.

Three consensus primers (two sense primers and 1 antisense primer labeled with dinitrophenyl) were used in a multiplex PCR assay for detection and typing of oncogenic and nononcogenic HPV types (23). The amplification products were hybridized with specific labeled oligoprobes mixed in two cocktails (oncogenic and nononcogenic biotinylated HPV oligoprobes) which could then be deposited in one well of streptavidin-coated microplates. PCR products were detected with anti-dinitrophenyl monoclonal antibody and horseradish peroxidase (PCR-enzyme immunoassay). The test was evaluated in cervical scrapings from 181 patients at high risk for cervical cancer selected because of their histories of cytological and/or histological cervical and vaginal abnormalities. These patients were classified with regard to the presence of lesions in cervical scrapings as none (i.e., no lesions; $n = 137$), low grade (i.e., few lesions $n = 20$), and high grade (i.e., many lesions $n = 24$). In patients without lesions, the multiplex PCR detected nononcogenic HPV in 29 patients (18.3%) and oncogenic HPV in 44 patients (32.1%), including 21 (15.3%) patients presenting with coinfection. In the low-grade group, oncogenic HPV was detected in 12 patients (60%) and nononcogenic HPV was detected in 5 patients (25%), including 4 (20%) cases of coinfection. In the high-grade patients, oncogenic HPVs were detected in 95.8% (23 patients) with coinfection with nononcogenic HPV in 4 of these patients (16.7%). The multiplex PCR was negative in scrapings from the remaining 93 patients (85 patients without lesions, 7 patients in the low-grade group, and 1 patient in the high-grade group). The authors concluded that the test is simple and reproducible and can be automated.

Another variation (75) combined a nested multiplex PCR, utilizing primers specific for low- or high-risk HPV types with restriction endonuclease analysis. The accuracy of this ap-

proach was confirmed by examining cervical scrapings from 44 patients. HPVs were detected and typed in scrapings from 7 patients with koilocytosis, 8 patients with dysplasia or metaplasia, 3 patients with condyloma acuminata, and 18 patients with cervical invasive neoplasia but in no samples from the seven healthy controls. More recently, a simple multiplex PCR utilizing three primer pairs for amplification of HPV-16, -18, and -33 in combination with a colorimetric microplate hybridization as a post-PCR detection system has been developed (35). The system uses three type-specific capture oligonucleotides linked covalently to a single microplate well and three type-specific multibiotinylated probes for detection. The methodology was evaluated using a total of 55 cervical smears and biopsy samples from 55 women with cervical lesions, resulting in 100% correlation with the results of other PCRs using consensus primers.

A multiplex PCR that combined the detection of *C. trachomatis* and two viruses (HPV and HSV) was developed, optimized, and evaluated using cervical and endocervical specimens from patients suspected to be infected with one or more of these agents (64). The test produced 100% correlation with the results of the uniplex PCRs in 92 genital swabs (29 were positive for HSV, 16 were positive for HPVs, and one was positive for *C. trachomatis*). In addition, a coinfection with HPV and HSV was detected by the multiplex PCR. In other studies (7, 66, 73), primer pairs for HSV were combined with those for *Haemophilus ducreyi* and *Treponema pallidum* to construct a multiplex PCR for diagnosis of genital ulceration. The PCR products were detected utilizing a colorimetric detection system in which three separate microwells containing immobilized oligonucleotide capture probes were used. In one of these studies (73), the sensitivity of this multiplex PCR in 298 genital ulcer swab specimens to detect HSV, *H. ducreyi*, and *T. pallidum* were 100, 98.4, and 91%, respectively, compared to 71.8, 74.2, and 81% by HSV culture, *H. ducreyi* culture, and darkfield microscopy for *T. pallidum*, respectively. The same multiplex PCR was used to evaluate swab specimens from 38 sequential patients with genital ulcer disease who received clinical diagnoses and syndromic treatment (7). These specimens were also tested for *H. ducreyi* by culture which was reported to be negative for all specimens tested. Of the 38 specimens, the multiplex PCR detected HSV in 31 specimens (81.6%) and HSV and *T. pallidum* in 1 specimen (2.3%) (coinfection). The test was negative in the remaining six specimens (15.8%). The clinical diagnoses corresponded poorly to the results of the multiplex PCR for chancroid and syphilis; none of the samples from the six suspected cases of chancroid and the one case of syphilis were positive for *H. ducreyi* and *T. pallidum*, respectively, by the multiplex PCR. Of the six clinically suspected cases of chancroid, the multiplex PCR was negative for all pathogens in one case and positive for HSV in the remaining five cases. Of 24 clinically suspected cases of genital herpes, 21 cases (87.5%) were confirmed by multiplex PCR.

Although clinical findings correlated poorly with multiplex PCR results, it is known that the clinical manifestations of all three infections can vary significantly (25). A few specimens produced negative results for which there may be several possible explanations, including ulcers due to trauma, low organism numbers, inadequate sampling techniques, and lesions due to other etiologic agents. These studies highlight the advantages of multiplex PCR over standard laboratory techniques and allow the detection of coinfection.

Ocular Infections

The benefits of PCR diagnostics over conventional techniques in the diagnosis of ocular infection are well documented for both the anterior and posterior segment of the eye (1, 24, 31, 80). The development and evaluation of multiplex PCR for detection of adenovirus, HSV, and *C. trachomatis* in cases of keratoconjunctivitis demonstrated the feasibility of simultaneous screening for these agents (49; A. C. Yeo, R. J. Cooper, D. J. Morris, and C. C. Storey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-416, 1997). These studies further highlight the difficulties of multiplex PCR and also provide substantial evidence for the importance of careful selection of oligonucleotide primers. In the study by Jackson et al. (49), a multiplex PCR was designed to detect adenovirus and HSV in eye swabs. The test produced results identical to those of virus isolation for 18 of 20 eye swabs (positive for adenovirus in five swabs, positive for HSV for five swabs and negative for adenovirus and HSV in eight swabs) but the remaining two specimens positive for adenovirus and HSV by virus isolation were negative by the multiplex PCR. However, the multiplex PCR proved superior to culture for the rapid diagnosis of viral keratoconjunctivitis. Replacement of the adenovirus primer pair to allow broader reactivity with adenovirus serotypes and inclusion of a primer pair targeting the cryptic plasmid of *C. trachomatis* (A. C. Yeo, R. J. Cooper, D. J. Morris, and C. C. Storey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-146, 1997) yielded a triplex multiplex PCR. The sensitivity of this adenovirus-HSV-*C. trachomatis* multiplex PCR in comparison to a uniplex PCR for the detection of adenovirus was 100%. However, the performance of the test to detect either HSV or *C. trachomatis* was relatively poor (69% compared to cell culture or 72% compared to an antigen detection technique). Selection of alternate HSV and *C. trachomatis* primer pairs allowed development of a multiplex PCR with identical sensitivity to that of uniplex PCRs for detection of each of the three targets.

Immunocompromised Patients

Due to the importance of HHV infections in immunocompromised patients, a number of authors have developed methods for the simultaneous detection of these viruses in various clinical specimens. Two different genes of CMV were detected with a single-step multiplex PCR in clinical specimens from renal transplant recipients and other CMV-seropositive patients (11). The test was sensitive and allowed monitoring of CMV infection by quantitation of CMV DNA. The latter was based on the assumption that samples with small amounts of viral DNA are more likely to lead to the amplification of only one of the two targets. Samples from which a single target is amplified contain on average sevenfold fewer viral genomes per 10^6 leukocytes than those from which both targets are amplified. This approach was evaluated on serial leukocyte DNA samples taken from 34 patients during the first 12 weeks post-renal transplantation, and it was concluded that findings of three consecutive tests in which both CMV targets were amplified were highly indicative of patients who had developed a very high load of CMV with a sensitivity of 100% and specificity of 88%. Thus, the protocol can be used for efficient identification of transplant recipients at risk of clinically significant infection. A similar PCR format was used to develop a multiplex PCR for amplification of four different regions of the CMV genome (61). The test sensitivity for detection of genomic cell DNA infected with CMV was similar to that obtained by each pair of the primers (uniplex PCRs). The test

proved to have a high diagnostic utility for detection of CMV variants with maximal sensitivity and specificity.

In heart transplant recipients a hot start nested multiplex PCR was used to evaluate the possible reactivation of HHV-6 and HHV-7 (67). The test was also coupled with CMV antigenemia assay and HSV isolation. The multiplex PCR was performed on buffy coat and plasma samples from the 21 recipients and on buffy coat samples from healthy blood donors. Whereas 12 of 21 (57.1%) heart transplant recipients showed CMV and/or HSV reactivation, HHV-6 was detected in 2 of 21 (9.5%) of the recipients and in 7 of 56 (12.5%) blood donors. HHV-7 DNA was detected in 13 of the 21 recipients (61.9%) and in 30 of 56 (56.6%) blood donors. One of the patients positive for HHV-6 and 10 of those positive for HHV-7 were positive for CMV. The clinical significance of these concurrent infections remains to be determined, but their detection further highlights the utility of multiplex PCR in investigation of transplant patients.

Detection and typing of all human lymphotropic herpesviruses (EBV, CMV, HHV-6 variants A and B, HHV-7, and HHV-8) utilizing primer pairs designed to amplify a highly conserved region within the DNA polymerase gene have also been described (77). The test also included an internal control (100 molecules of a cloned fragment of the porcine pseudorabies herpesvirus genome) for detection of false-negative results. The test revealed a sensitivity of 10 to 100 molecules of each virus DNA and produced a 100% correlation in a total of 35 well-characterized specimens in which one of these viruses was known to be present, including Kaposi's sarcoma skin lesions and serum, CSF, saliva, and urine samples.

Simultaneous detection of EBV and the protozoa *Toxoplasma gondii* in CSFs of AIDS patients with EBV-associated primary nervous system lymphoma and toxoplasma encephalitis has been described (87). This multiplex PCR detected EBV DNA in 9 of 14 patients with CNS lymphoma and in 2 of 38 patients without disease and *T. gondii* DNA in 8 of 8 patients with toxoplasma encephalitis but in none without toxoplasmosis.

The major problem with the use of PCR in specimens from immunocompromised patients is the relationship of positive results to clinical disease. This is particularly difficult with herpesviruses, because positive PCR results may not always be accompanied by signs or symptoms in the patient. This can leave the clinician in a dilemma of whether or not to treat. Two approaches have been used to address this problem. Quantitative PCR can be used to measure viral load with the expectation that a high viral load is likely to herald clinical disease. The other approach is to test sequential specimens and only recommend treatment if positive results persist rather than responding to what may be a transient reactivation of a herpesvirus. Multiplex PCR may be readily applied to the latter situation, but multiplex quantitative PCR remains a formidable technological challenge.

Other Applications

A number of studies have utilized multiplex PCRs for detection and differentiation of human retroviruses (45, 46, 99, 111). Four primer pairs were combined to detect the *gag* region of HIV type 1 (HIV-1), the *env* region of HIV-2, the *pol* region of human T-cell leukemia virus type 1 (HTLV-1), and the *tax* region of HTLV-2 (45). Amplicons were detected by liquid hybridization using ^{32}P-end-labeled oligonucleotides. In the evaluation of a serologically well-established panel of singly and dually infected individuals, the assay detected 21 of 22 HIV-1, 8 of 10 HIV-2, 8 of 8 HTLV-1, and 8 of 8 HTLV-2 infections. The test was as sensitive as uniplex PCRs and allowed the detection of coinfection. Sunzeri et al. (99) developed a multiplex PCR utilizing primer pairs targeting a portion of the *gag* region of HIV-1, the *pol* gene of HTLV-1 and -2, and a region of the HLA-DQ-α locus as an internal control. Products were analyzed by automated capillary DNA chromatography (products can also be separated and visualized using gel electrophoresis and ethidium bromide staining). The test detected as few as 1 to 10 infected cells (2 to 20 target sequences) and was as sensitive as uniplex PCRs.

Several studies have highlighted the potential use of PCR in screening donated blood for transfusion-transmitted viruses (48), but the need for multiple, discrete PCR assays for achieving this purpose has restricted such an application. Uniplex PCR would be an impractical approach for rapid screening of hundreds of specimens per day for a range of transfusion-transmitted viruses. Multiplex PCR presents a more practicable solution to the problem, and the methodology is sensitive enough to diagnose silent (serologically negative) carriers of viruses such as HIV, HTLV, and other nonretroviral transfusion-transmitted viruses, including hepatitis B virus (HBV), HCV, and CMV.

Multiplex PCR offers a cost-effective solution which, with refinement and full automation would allow screening of the donated blood supply. Indeed, a recent study (106) demonstrated the promise of multiplex PCR automation for both rapid and reliable screening of transfusion-transmitted viruses in donated blood and transplantable tissues. A hot start multiplex PCR was developed to identify and determine the abundance of HIV-1, HIV-2, and HTLV-1 and -2. Viral DNA sequences were amplified in a single reaction, and the resulting amplicons were detected in real time by the hybridization of four differently colored, amplicon-specific detector probes called molecular beacons present within the same reaction tube. The color of the fluorescence produced during the amplification process identified the retrovirus present in the sample, and correlation of the thermal cycles required with the intensity of each fluorescent signal developed provides an accurate measure of the number of virus sequences present in the original sample. The test had a sensitivity of 10 retroviral genomes in the presence of 100,000 copies of another retrovirus, and up to 96 samples can be analyzed in 3 h on a single plate. The test was also evaluated using 43 human blood samples known to contain human retroviruses and produced 100% agreement in 11 samples positive for HIV-1, 4 samples positive for HIV-2, 15 samples positive for HTLV-1, and 17 samples positive for HTLV-2, and the 10 control samples remained negative for all targets.

Multiplex PCRs for other transfusion-transmitted viruses have also appeared in the literature. A multiplex PCR which detects both HBV and HBC genomic sequences in serum samples has been developed (71). The test is carried out in two stages. HCV RNA is first reverse transcribed into cDNA, and both HCV cDNA and HBV DNA are then coamplified using primers that target conserved sequences from both viruses. The test was applied to sera from nine donors, of which seven were positive for HBsAg, anti-HBc, and anti-HCV; one was reactive for both anti-HCV and anti-HBc; and one was reactive for both HBsAg and anti-HBc. The multiplex PCR produced results confirming the presence of both HBV- and HCV-specific genomic sequences in eight of eight sera reactive for the serological markers of both viruses and also in a serum that was reactive for HBV markers only. The method was modified to include primers that target the HCV 5′ untranslated region and HBV pre-S and S region in a one-step multiplex PCR method. This modification allowed a simple simultaneous am-

plification of both viruses with 100% concordance with its respective uniplex PCRs (47).

HCV and GB virus type C (GBV-C)/HGV genomes in plasma samples from transfused subjects have also been amplified by a multiplex PCR (16). The test was evaluated retrospectively in 50 plasma samples in comparison with the results of serology. Of the 50 samples, 40 were positive for anti-HCV and 10 samples remained antibody negative. The multiplex PCR and the corresponding uniplex PCR produced identical results, being positive for HCV RNA in 32 samples of the 40 anti-HCV positive samples. GBV-C RNA was detected in 5 of the 32 HCV-positive samples (coinfection) and in 2 of 10 samples that were anti-HCV negative. More widespread application of multiplex PCR aimed at detecting transfusion-transmitted viruses and/or other pathogens will demand thorough optimization and full automation of procedures to avoid the production of both false-positive and false-negative results. The demands of transfusion medicine of high-speed, high-throughput screening will place great technical demands upon these procedures.

Multiplex PCR methodology has also proven to be a valuable tool for differentiation, subgrouping, subtyping, and genotyping of viruses (10, 29, 78, 84, 121). Differentiation of polioviruses from nonpoliovirus enteroviruses in both clinical (stool) and environmental (sewage) specimens has been feasible using RT-multiplex PCR (29). Stool or sewage specimens are first inoculated onto cell cultures in tubes, and after overnight incubation the cultures are subjected to RT-multiplex PCR. A primer pair detecting all enteroviruses in the presence of another two primer pairs specific for the polioviruses was tested. The enterovirus-specific primer pair generated a product size of 300 bp, and the poliovirus-specific primer pairs generated three different PCR products of 200, 600, and 1,000 bp, assuring easy identification on agarose gels. The result is interpreted as "nonpolio enterovirus" if only the enterovirus-specific PCR product (300 bp) is observed; as "poliovirus" if both the enterovirus-specific product (300 bp) and at least one of the polio-specific products are observed (200, 600, and 1,000 bp); or as "no enterovirus" if the multiplex PCR remains negative for all products. A total of 36 poliovirus strains produced the expected results by the multiplex PCR, consistent with polioviruses, and only one isolate (coxsackievirus A21) of 45 nonpoliovirus strains demonstrated a band with a poliovirus-specific primer pair. The protocol revealed a sensitivity of 3 PFU, could be interpreted within 24 h, and was highly insensitive to substances in the sample (stool and sewage) which inhibit cell culture isolation.

A multiplex RT-PCR that simultaneously identifies Sabin poliovirus types 1, 2, and 3 vaccine strains has been described (10). This hot start-based multiplex PCR was a modification of a procedure previously described (121). The assay utilized three serotype-specific primer sets that map to the region of the poliovirus genome encoding the amino terminus of the VP1 capsid protein, a region known for its heterogeneity among the three Sabin poliovirus serotypes. In a total of 195 stool samples collected from 26 vaccinees following administration of the first dose of the trivalent oral vaccine, the multiplex PCR was more sensitive than culture for the detection of poliovirus types 1, 2, and 3. The percentages of specimens positive by the multiplex PCR for serotypes 1, 2, and 3 were 67.2, 82.6, and 53.8%, respectively, compared to 55.4, 64.1, and 27.7% by virus isolation. The duration of recovery of positive samples by PCR varied according to serotype: 4 to 8 weeks for type 2 and 1 to 8 weeks for types 1 and 3, although poliovirus type 3 shedding ceased in approximately 70% of vaccinees within a week after immunization. This modified multiplex PCR allows direct characterization of the virus in stool specimens without cell culture—a process which may, through the selection of genetic variants, not accurately represent the virus population in the original specimens.

Human adenoviruses in clinical samples were detected with six primer pairs specific for all adenovirus subgenera (A to F) (78). Each primer pair consists of a primer derived from the subgenus-specific sequences and a primer that targets a conserved hexon region, obviating the need for restriction endonuclease analysis. The six subgenus-specific amplicons were distinguishable by agarose gel electrophoresis as products of 299, 465, 269, 331, 399, and 586 bp representing, respectively, those of adenovirus subgroup A to F. The test revealed a detection limit of a single copy of adenovirus DNA. The primer pairs produced 100% specificity when evaluated on 23 adenovirus prototypes, representing all six subgenera; on 9 intermediate strains from subgenera B and D; and on 16 subgenus C genome types. In clinical specimens, the test was positive for adenovirus in 26 of 65 stool specimens (4 samples belonging to subgroup A, 2 samples belonging to subgroup B, 6 samples belonging to subgroup C, 13 samples belonging to subgroup F, and 1 sample showing coinfection with subgroup C and F), in 13 of 23 eye specimens (1 sample belonging to subgroup B, 10 samples belonging to subgroup D, and 2 samples belonging to subgroup E), and 2 of 12 throat specimens (belonging to adenovirus subgroup B). Of significance, the test has clinical value as can be highlighted by its discrimination of adenovirus subgroup D, which causes the severe and highly contagious epidemic keratoconjunctivitis, from subgroup B and E adenoviruses, which may cause relatively mild ocular infections. The test could also facilitate the primary classification of unknown virus isolates.

Multiplex PCR has also proved to be a valuable tool for genotyping HBV strains (84). The first round of amplification utilizes a primer pair to amplify the entire pre-S region of the virus genome. Within the pre-S region nucleotide exchanges are observed that are partly correlated to the serological surface antigen subtypes. In the second round of amplification, five additional subtype-specific primers and two universal non-group-specific primers are added to generate two to four DNA fragments of defined sizes indicative for the subtype. The method proved to be a useful epidemiological tool for studying HBV transmission and may be adapted to genomes of other infectious agents demonstrating a suitable degree of sequence variability.

Other applications of multiplex PCR include maximizing the inclusivity of PCR-based assays to detect viral strains (11, 51, 61, 93), exploring genetic reassortment among viruses (90), studying association of virus with disease (105), studying virus pathogenesis (6), exploring mechanisms of virus evasion and interference with the host's immune response (41), and facilitating the detection and characterization of viruses and other pathogens retrospectively in diverse archival specimens of limited volumes (2). In addition, the methodology has proved to be a powerful tool for characterization of nonhuman viruses (50, 70, 85, 104, 114, 116).

CONCLUSION AND PERSPECTIVES

Optimization of multiplex PCRs can prove difficult. A stepwise matrix-style approach may be followed; i.e., a number of optimal primer pairs are combined and the combination giving the best result is then chosen to be optimized or evaluated in a multiplex PCR format. Alterations of other PCR components over those usually described for most uniplex PCRs have rarely improved the efficiency of the test. Recent developments

in PCR technology, however, may facilitate the development of multiplex PCRs. The most appropriate of these seems to be the use of the nonmechanical hot start PCR (8, 53).

Thorough evaluation and validation of new multiplex PCR procedures is essential. The sensitivity and specificity must be thoroughly evaluated using standardized, purified nucleic acids. Where available, full use should be made of external quality control materials, and both external and internal quality controls must be rigorously applied. These must include the provision of both negative control specimens and, for each nucleic acid target, a positive control designed to ensure early signalling of any reduction in test sensitivity from assay to assay. As the number of microbial agents detectable by PCR increases, it will become highly desirable for practical purposes to achieve simultaneous detection of multiple agents that cause similar or identical clinical syndromes and/or share similar epidemiological features. In addition, attention should be given to primer pairs detecting multiple strains or types to ensure the identification of as many strains of the target species as possible. Where possible, robust (i.e., reliable in routine diagnostic settings) multiplex PCRs that do not use nested primers are preferable to avoid contamination (122), to rationalize use in routine settings, and to facilitate automation (110).

Commercial development of PCR has facilitated the widespread introduction of this procedure and improved both the reliability and ease of use of the technology. Commercially available applications of multiplex PCR are as yet in their infancy. While many commercially available PCRs include either internal control molecules or reporter molecules as internal standards within the test, the primer binding targets of both internal control and target nucleic acids are the same. Thus, although two molecules are amplified in the PCR, this is not true multiplexing. To date a multiplex PCR for *Neisseria gonorrhoea* and *C. trachomatis* represents the sole commercial multiplex PCR from Roche Diagnostics, and Argene Biosoft (Ariége, France) produces a multiplex PCR for detection of HHVs. Undoubtedly, many more commercial applications of multiplex PCR may be anticipated, and when coupled with developments in microelectronic detection devices (58) the prospect of extralaboratory "at-the-bedside" multiplex PCR testing may be envisaged. Given the advantages already demonstrated by the use of multiplex PCR along with the recent developments in this technology, future applications of PCR, when possible, should be aimed at constructing multiple detection systems in which a number of clinically and epidemiologically relevant pathogens (viruses, bacteria, parasites, and/or fungi) may be detected, characterized, and/or inevitably uncovered in a symptom- and/or system-specific manner.

REFERENCES

1. **Adleberg, J. M., and C. Wittwer.** 1995. Use of the polymerase chain reaction in the diagnosis of ocular disease. Curr. Opin. Ophthalmol. **6**:80–85.
2. **Bakaletz, L. O., G. J. White, J. C. Post, and G. D. Ehrlich.** 1998. Blinded multiplex PCR analyses of middle ear and nasopharyngeal fluid from chinchilla models of single- and mixed-pathogen-induced otitis media. Clin. Diagn. Lab. Immunol. **5**:219–224.
3. **Beards, G., C. Graham, and D. Pillay.** 1998. Investigation of vesicular rashes for HSV and HZV by PCR. J. Med. Virol. **54**:155–157.
4. **Bej, A. K., M. H. Mahbubani, and R. M. Atlas.** 1991. Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and applications. Crit. Rev. Biochem. Mol. Biol. **26**:301–334.
5. **Belli, A., B. Rodriguez, H. Aviles, and E. Harris.** 1998. Simplified polymerase chain reaction detection of new world *Leishmania* in clinical specimens of cutaneous leishmaniasis. Am. J. Trop. Med. Hyg. **58**:102–109.
6. **Bertram, S., F. T. Hufert, J. van Lunzen, and D. von Laer.** 1996. Coinfection of individual leukocytes with human cytomegalovirus and human immunodeficiency virus is a rare event *in vivo*. J. Med. Virol. **49**:283–288.
7. **Beyrer, C., K. Jitwatcharanan, C. Natpratan, R. Kaewvichit, K. E. Nelson, C. Y. Chen, J. B. Weiss, and S. A. Morse.** 1998. Molecular methods for the diagnosis of genital ulcer disease in a sexually transmitted disease clinic population in Northern Thailand: predominance of the herpes simplex virus infection. J. Infect. Dis. **178**:243–246.
8. **Birch, D. E., L. Kolmodin, J. Wong, G. A. Zangenberg, M. A. Zoccoli, N. McKinney, and K. K. Y. Young.** 1996. Simplified hot start PCR. Nature **381**:445–446.
9. **Brownie, J., S. Shawcross, J. Theaker, D. Whitcombe, R. Ferrie, C. Newton, and S. Little.** 1997. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Res. **25**:3235–3241.
10. **Buonagurio, D. A., J. W. Coleman, S. A. Patibandla, B. S. Prabhakar, and J. M. Tatem.** 1999. Direct detection of Sabin poliovirus vaccine strains in stool specimens of first-dose vaccinees by a sensitive reverse transcription-PCR method. J. Clin. Microbiol. **37**:283–289.
11. **Caballero, O. L., C. L. P. Menezes, M. C. S. L. Costa, S. C. Fernandes, T. M. Anacleto, R. M. De Oliveira, E. A. Viotti, E. R. F. Tavora, S. S. Vilaca, E. Sabbaga, F. J. De Paula, P. F. Tavora, L. L. Villa, and A. J. G. Simpson.** 1997. Highly sensitive single-step PCR protocol for diagnosis and monitoring of human cytomegalovirus infection in renal transplant recipients. J. Clin. Microbiol. **35**:3192–3197.
12. **Casas, I., A. Tenorio, J. M. Echevarria, P. E. Klapper, and G. M. Cleator.** 1997. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. J. Virol. Methods **66**:39–50.
13. **Casas, I., F. Pozo, G. Trallero, J. M. Echevarria, and A. Tenorio.** 1999. Viral diagnosis of neurological infection by RT multiplex PCR: a search for entero- and herpesviruses in a prospective study. J. Med. Virol. **57**:145–151.
14. **Cassinotti, P., and G. Siegl.** 1998. A nested-PCR assay for the simultaneous amplification of HSV-1, HSV-2, and HCMV genomes in patients with presumed herpetic CNS infection. J. Virol. Methods **71**:105–114.
15. **Cassinotti, P., H. Mietz, and G. Siegl.** 1996. Suitability and clinical application of a multiplex nested PCR assay for the diagnosis of herpes simplex virus infections. J. Med. Virol. **50**:75–81.
16. **Caudai, C., M. G. Padula, V. Bettini, and P. E. Valensin.** 1998. Detection of HCV and GBV-C/HGV infection by multiplex PCR in plasma samples of transfused subjects. J. Virol. Methods **70**:79–83.
17. **Cha, R. S., and W. G. Thilly.** 1993. Specificity, efficiency, and fidelity of PCR. PCR Methods Appl. **3**:S18–S29.
18. **Chamberlain, J. S., and J. R. Chamberlain.** 1994. Optimization of multiplex PCRs, p. 38–46. *In* K. B. Mullis, F. Ferre, and R. A. Gibbs (ed.), The polymerase chain reaction. Birkhauser, Boston, Mass.
19. **Chamberlain, J. S., R. A. Gibbs, J. E. Ranier, P. N. Nguyen, and C. T. Caskey.** 1989. Multiplex PCR for the diagnosis of Duchenne muscular dystrophy, p. 272–281. *In* D. H. Gelfand, M. A. Innis, J. J. Shinsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
20. **Chamberlain, J. S., R. A. Gibbs, J. E. Ranier, P. N. Nguyen, and C. T. Caskey.** 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. **16**:11141–11156.
21. **Chou, Q., M. Russel, D. E. Birch, J. Raymond, and W. Bloch.** 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. **11**:1717–1723.
22. **Cinque, P., L. Vago, R. Marenzi, B. Giudici, T. Weber, R. Corradini, D. Ceresa, A. Lazzarin, and A. Linde.** 1998. Herpes simplex virus infections of the central nervous system in human immunodeficiency virus-infected patients: clinical management by polymerase chain reaction assay of cerebrospinal fluid. Clin. Infect. Dis. **27**:303–309.
23. **Clavel, C., S. Rihet, M. Masure, C. Chypre, J. C. Boulanger, C. Quereux, and P. Birembaut.** 1998. DNA-EIA to detect high and low risk HPV genotypes in cervical lesions with E6/E7 primer mediated multiplex PCR. J. Clin. Pathol. **51**:38–43.
24. **Della, N. G.** 1996. Molecular biology in ophthalmology: a review of principles and recent advances. Arch. Ophthalmol. **114**:457–463.
25. **DiCarlo, R. P., and D. H. Martin.** 1997. The clinical diagnosis of genital ulcer disease in men. Clin. Infect. Dis. **25**:292–298.
26. **Dieffenbach, C. W., T. M. J. Lowe, and G. S. Dveksler.** 1993. General concepts for PCR primer design. PCR Methods Appl. **3**:S30–S37.
27. **Echevarria, J. E., D. D. Erdman, E. M. Swierkosz, B. P. Holloway, and L. J. Anderson.** 1998. Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. J. Clin. Microbiol. **36**:1388–1391.
28. **Edwards, M. C., and R. A. Gibbs.** 1994. Multiplex PCR: advantages, development, and applications. PCR Methods Appl. **3**:S65–S75.
29. **Egger, D., L. Pasamontes, M. Ostermayer, and K. Bienz.** 1995. Reverse transcription multiplex PCR for differentiation between polio and enteroviruses from clinical and environmental samples. J. Clin. Microbiol. **33**: 1442–1447.
30. **Ellis, L., D. M. Fleming, and M. C. Zambon.** 1997. Multiplex reverse transcription-PCR for surveillance of influenza A and B viruses in England and Wales in 1995 and 1996. J. Clin. Microbiol. **35**:2076–2082.
31. **Elnifro, E. M., R. J. Cooper, P. E. Klapper, A. S. Bailey, and A. B. Tullo.** 1999. Diagnosis of viral chlamydial keratoconjunctivitis: which laboratory test? Br. J. Ophthalmol. **83**:622–627.
32. **Emmanuel, P. J.** 1993. Polymerase chain reaction from bench to bedside.

Applications for infectious disease. J. Fla. Med. Assoc. 80:627–630.
33. **Fan, J., K. J. Henrickson, and L. L. Savatski.** 1998. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction enzyme hybridization assay (Hexaplex). Clin. Infect. Dis. 26:1397–1402.
34. **Garcia, L., J. C. Tercero, B. Legido, J. A. Ramos, J. Alemany, and M. Sanz.** 1998. Rapid detection of *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromona gingivalis* by multiplex PCR. J. Periodontal Res. 33:59–64.
35. **Godfroid, E., M. Heinderyckx, F. Mansy, I. Fayt, J. C. Noel, L. Thiry, and A. Bollen.** 1998. Detection and identification of human papilloma viral DNA, types 16, 18, and 33 by a combination of polymerase chain reaction and a colorimetric solid phase capture hybridisation assay. J. Virol. Methods 75:69–81.
36. **Grondahl, B., W. Puppe, A. Hoppe, I. Khne, J. A. L. Weigl, and H. J. Schmitt.** 1999. Rapid identification of nine microorganisms causing acute respiratory tract infection by single-tube multiplex reverse transcription-PCR: feasibility study. J. Clin. Microbiol. 37:1–7.
37. **Harmon, K. M., G. M. Ransom, and I. V. Wesley.** 1997. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. Mol. Cell. Probes 11:195–200.
38. **Harris, E.** 1996. Appropriate transfer of molecular technology. Asia Pacific Technol. Monitor 13:29–33.
39. **Harris, E., G. Kropp, A. Belli, B. Rodriguez, and N. Agabian.** 1998. Single-step multiplex PCR assay for characterization of New World *Leishmania* complexes. J. Clin. Microbiol. 36:1989–1995.
40. **Harrison, T. J.** 1998. The polymerase chain reaction—a time of transition from research to routine. J. Clin. Pathol. 51:491–492.
41. **Hassan-Walker, A. F., A. V. Cope, P. D. Griffiths, and V. C. Emery.** 1998. Transcription of the human cytomegalovirus natural killer decoy gene, UL18, *in vitro* and *in vivo*. J. Gen. Virol. 79:2113–2116.
42. **Hendolin, P. H., A. Markkanen, J. Ylikoski, and J. J. Wahlfors.** 1997. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. J. Clin. Microbiol. 35:2854–2858.
43. **Henegariu, O., N. A. Heerema, S. R. Dlouhy, G. H. Vance, and P. H. Vogt.** 1997. Multiplex PCR: critical parameters and step-by-step protocol. BioTechniques 23:504–511.
44. **Hengen, P. N.** 1997. Optimizing multiplex and LA-PCR with betaine. Trends Biol. Sci. 22:225–226.
45. **Heredia, A., V. Soriano, S. H. Weiss, R. Bravo, A. Vallejo, T. N. Denny, J. S. Epstein, and I. K. Hewlett.** 1996. Development of a multiplex PCR assay for the simultaneous detection and discrimination of HIV-1, HIV-2, HTLV-I and HTLV-II. Clin. Diagn. Virol. 7:85–92.
46. **Hewlett, I. K., M. Ruta, K. Cristiano, C. A. Hawthorne, and J. S. Epstein.** 1989. Co-amplification of multiple regions of the HIV-1 genome by the polymerase chain reaction: potential use in multiple diagnosis. Oncogene 4:1149.
47. **Hu, K. O., C. H. Yu, S. Lee, F. G. Villamil, and J. M. Vierling.** 1995. Simultaneous detection of both hepatitis B virus DNA and hepatitis C virus RNA using a combined one-step polymerase chain reaction technique. Hepatology 21:901–907.
48. **Jackson, J. B.** 1990. Polymerase chain reaction in transfusion medicine. Transfusion 30:51.
49. **Jackson, R., D. J. Morris, R. J. Cooper, A. S. Bailey, P. E. Klapper, and G. M. Cleator.** 1996. Multiplex polymerase chain reaction for adenovirus and herpes simplex virus in eye swabs. J. Virol. Methods 56:41–48.
50. **Jacobi, V., G. D. Bachand, R. C. Hamelin, and J. D. Castello.** 1998. Development of a multiplex immunocapture RT-PCR assay for detection and differentiation of tomato and tobacco mosaic tobamoviruses. J. Virol. Methods 74:167–178.
51. **Jin, L., A. Richards, and D. W. G. Brown.** 1996. Development of a dual target-PCR for detection and characterization of measles virus in clinical specimens. Mol. Cell. Probes 10:191–200.
52. **Kaucner, C., and T. Stinear.** 1998. Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. App. Environ. Microbiol. 64:1743–1749.
53. **Kebelmann-Betzing, C., K. Seeger, S. Dragon, G. Schmitt, A. Moricke, T. A. Schild, G. Henze, and B. Beyermann.** 1998. Advantages of a new *Taq* DNA polymerase in multiplex PCR and time-release PCR. BioTechniques 24: 154–158.
54. **Klapper, P. E., D. L. Jungkind, T. Fenner, R. Antinozzi, J. Schirm, and C. Blanckmeister.** 1998. Multicenter international work flow study of an automated polymerase chain reaction instrument. Clin. Chem. 44:1737–1739.
55. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature 339:237–238.
56. **Lazarus, P., and S. Caruana.** 1996. Typing of common human papilloma virus strains by multiplex PCR. Anal. Biochem. 243:198–201.
57. **Linz, U., U. Delling, and H. Rübsamen-Waigmann.** 1990. Systematic studies on parameters influencing the performance of the polymerase chain reaction. J. Clin. Chem. Clin. Biochem. 28:5–13.
58. **Livache, T., H. Bazin, and G. Mathis.** 1998. Conducting polymers on microelectronic devices as tools for biological analyses. Clin. Chim. Acta 278:171–176.
59. **Mahony, J. B., D. Jang, S. Chong, K. E. Luinstra, J. W. Sellors, M. Tyndall, and M. Chernesky.** 1997. Detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, and *Mycoplasma genitalium* in first-void urine specimens by multiplex polymerase chain reaction. Mol. Diagn. 2:161–168.
60. **Mahony, J. B., K. E. Luinstra, M. Tyndall, J. W. Sellors, J. Krepel, and M. Chernesky.** 1995. Multiplex PCR for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genitourinary specimens. J. Clin. Microbiol. 33:3049–3053.
61. **Markoulatos, P., V. Samara, N. Siafakas, E. Plakokefalos, N. Spyrou, and M. L. Moncany.** 1999. Development of a quadriplex polymerase chain reaction for human cytomegalovirus detection. J. Clin. Lab. Anal. 13:99–105.
62. **McElhinney, L. M., R. J. Cooper, and D. J. Morris.** 1995. Multiplex polymerase chain reaction for human herpesvirus-6, human cytomegalovirus, and human β-globin DNA. J. Virol. Methods 53:223–233.
63. **Minjolle, S., C. Michelet, I. Jusselin, M. Joannes, F. Cartier, and R. Collimon.** 1999. Amplification of the six major human herpesviruses from cerebrospinal fluid by a single PCR. J. Clin. Microbiol. 37:950–953.
64. **Mitrani-Rosenbaum, S., R. Tsvieli, O. Lavie, R. Boldes, E. Anteby, S. Shimonovitch, T. Lazarovitch, and A. Friedmann.** 1994. Simultaneous detection of three common sexually transmitted agents by polymerase chain reaction. Am. J. Obstet. Gynecol. 171:784–790.
65. **Mitsuhashi, M.** 1996. Technical report: part 2. Basic requirements for designing optimal PCR primers. J. Clin. Lab. Anal. 10:285–293.
66. **Morse, S. A., D. L. Trees, Y. Htun, F. Radebe, K. A. Orle, Y. Dangor, C. M. Beck-Sague, S. Schmid, G. Fehler, J. B. Weiss, and R. C. Ballard.** 1997. Comparison of clinical diagnosis and standard laboratory and molecular methods for the diagnosis of genital ulcer disease in Lesotho: association with human immunodeficiency virus infection. J. Infect. Dis. 175:583–589.
67. **Moschettini, D., A. De Milito, M. Catucci, A. Marconi, C. Rinina, M. L. Bianchi-Bandinelli, and P. E. Valensin.** 1998. Detection of human herpesvirus 6 and 7 in heart transplant recipients by multiplex polymerase chain reaction method. Eur. J. Clin. Microbiol. Infect. Dis. 17:117–119.
68. **Mutter, G. L., and K. A. Boynton.** 1995. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acids Res. 23:1411–1418.
69. **Myeroson, D., P. A. Lingenfelter, C. A. Gleaves, J. D. Meyers, and R. A. Bowden.** 1993. Diagnosis of cytomegalovirus pneumonia by polymerase chain reaction with archived frozen lung tissue and bronchoalveolar lavage fluid. Am. J. Clin. Pathol. 100:407–413.
70. **Nadin-Davis, S. A., W. Huang, and A. I. Wandeler.** 1996. The design of strain-specific polymerase chain reaction for discrimination of the raccoon rabies virus strain from indigenous rabies viruses of Ontario. J. Virol. Methods 57:1–14.
71. **Nedjar, S., F. Mitchell, and R. Biswas.** 1994. Simultaneous amplification and detection of specific hepatitis B virus and hepatitis C virus genomic sequences in serum samples. J. Med. Virol. 42:212–216.
72. **Nicodème, P., and J. Steyaert.** 1997. Selecting optimal oligonucleotide primers for multiplex PCR. Intelligent Syst. Mol. Biol. 51:210–213.
73. **Orle, K. A., C. A. Gates, D. H. Martin, B. A. Body, and J. B. Weiss.** 1996. Simultaneous PCR detection of *Haemophilus ducreyi*, *Treponema pallidum*, and herpes simplex virus types 1 and 2 from genital ulcers. J. Clin. Microbiol. 34:49–54.
74. **Osiowy, C.** 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol. 36:3149–3154.
75. **Pizzighella, S., G. Pisoni, F. Bevilacqua, and A. Vaona.** 1995. Simultaneous polymerase chain reaction detection and restriction typing for the diagnosis of human genital papillomavirus infection. J. Virol. Methods 55:245–256.
76. **Polz, M. F., and C. M. Cavanaugh.** 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64:3724–3730.
77. **Pozo, F., and A. Tenorio.** 1999. Detection and typing of lymphotropic herpesviruses by multiplex polymerase chain reaction. J. Virol. Methods 79:9–19.
78. **Pring-Akerblom, P., F. E. J. Trijssenaar, T. Adrian, and H. Hoyer.** 1999. Multiplex polymerase chain reaction for subgenus-specific detection of human adenovirus in clinical samples. J. Med. Virol. 58:87–92.
79. **Raeymaekers, L.** 1995. A commentary on the practical applications of competitive PCR. Genome Res. 5:91–94.
80. **Rajeev, B., and J. Biswas.** 1998. Molecular biologic techniques in ophthalmic pathology. Indian J. Ophthalmol. 46:3–13.
81. **Read, S. J., and J. B. Kurtz.** 1999. Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay. J. Clin. Microbiol. 37:1352–1355.
82. **Read, S. J., K. J. M. Jeffery, and C. R. M. Bangham.** 1997. Aseptic meningitis and encephalitis: the role of PCR in the diagnostic laboratory. J. Clin. Microbiol. 35:691–696.
83. **Rees, W. A., T. D. Yager, J. Korte, and P. H. von Hippel.** 1993. Betaine can

eliminate the base pair composition dependence of DNA melting. Biochemistry 32:137–144.
84. **Repp, R., S. Rhiel, K. H. Heermann, S. Schaefer, C. Keller, P. Ndumbe, F. Lampert, and W. H. Gerlich.** 1993. Genotyping by multiplex polymerase chain reaction for detection of endemic hepatitis B virus transmission. J. Clin. Microbiol. 31:1095–1102.
85. **Reubel, G. H., B. S. Crabb, and M. J. Studdert.** 1995. Diagnosis of equine gammaherpesvirus 2 and 5 infections by polymerase chain reaction. Arch. Virol. 140:1049–1060.
86. **Rithidech, K. N., J. J. Dunn, and C. R. Gordon.** 1997. Combining multiplex and touchdown PCR to screen murine microsatellite polymorphisms. BioTechniques 23:36–45.
87. **Roberts, T. C., and G. A. Storch.** 1997. Multiplex PCR for diagnosis of AIDS-related central nervous system lymphoma and toxoplasmosis. J. Clin. Microbiol. 35:268–269.
88. **Robertson, J. M., and J. Walsh-Weller.** 1998. An introduction to PCR primer design and optimisation of amplification reactions. Methods Mol. Biol. 98:121–154.
89. **Rochelle, P. A., R. D. Leon, M. H. Stewart, and R. L. Wolfe.** 1997. Comparison of primers and optimization of PCR conditions for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water. J. Clin. Microbiol. 63:106–114.
90. **Rodriguez, L. L., J. H. Owens, C. J. Peters, and S. T. Nichol.** 1998. Genetic reassortment among viruses causing hantavirus pulmonary syndrome. Virology 242:99–106.
91. **Roux, K. H.** 1995. Optimization and troubleshooting in PCR. PCR Methods Appl. 4:S185–S194.
92. **Ruano, G., D. E. Brash, and K. K. Kidd.** 1991. PCR: the first few cycles. Amplifications 7:1–4.
93. **Sevall, J. S.** 1990. Detection of parvovirus B19 by dot-blot and polymerase chain reaction. Mol. Cell. Probes 4:237–246.
94. **Sherlock, J., V. Cirigliano, M. Petrou, B. Tutschek, and M. Adinolfi.** 1998. Assessment of diagnostic quantitative fluorescent multiplex polymerase chain reaction assays performed on single cells. Ann. Hum. Genet. 62:9–23.
95. **Shuber, A. P., V. J. Grondin, and K. W. Klinger.** 1995. A simplified procedure for developing multiplex PCRs. Genome Res. 5:488–493.
96. **Shuber, A. P., J. Skoletsky, R. Stern, and B. L. Handelin.** 1993. Efficient 12-mutation testing in the CFTR gene: a general model for complex mutation analysis. Hum. Mol. Genet. 2:153–158.
97. **Soler, C., P. Allibert, Y. Chardonnet, P. Cros, B. Mandrand, and J. Thivolet.** 1991. Detection of human papillomavirus types 6, 11, 16 and 18 in mucosal and cutaneous lesions by the multiplex polymerase chain reaction. J. Virol. Methods 35:143–157.
98. **Stockton, J., J. S. Ellis, M. Saville, J. P. Clewley, and M. C. Zambon.** 1998. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J. Clin. Microbiol. 36:2990–2995.
99. **Sunzeri, F. J., T. H. Lee, R. G. Bronlee, and M. P. Busch.** 1991. Rapid simultaneous detection of multiple retroviral DNA sequences using the polymerase chain reaction and capillary DNA chromatography. Blood 77: 879–886.
100. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. App. Environ. Microbiol. 62:625–630.
101. **Taylor, G. R., and P. Robinson.** 1998. The polymerase chain reaction: from functional genomics to high-school practical classes. Curr. Opin. Biotechnol. 9:35–42.
102. **Tenorio, A., J. E. Echevarria, I. Casas, J. M. Echevarria, and E. Tabares.** 1993. Detection and typing of human herpesviruses by multiplex polymerase chain reaction. J. Virol. Methods 44:261–269.
103. **Vandenvelde, C., M. Verstraete, and D. Van Beers.** 1990. Fast multiplex polymerase chain reaction on boiled clinical samples for rapid viral diagnosis. J. Virol. Methods 30:215–227.
104. **Vangrysperre, W., and K. De Clercq.** 1996. Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. Arch. Virol. 141:331–344.
105. **Vesy, C. J., J. K. Greenson, A. C. Papp, P. J. Snyder, S. J. Qualman, and T. W. Prior.** 1993. Evaluation of celiac disease biopsies for adenovirus 12 and using a multiplex polymerase chain reaction. Mod. Pathol. 6:61–64.
106. **Vet, J. A. M., A. R. Majithia, S. A. E. Earras, S. Tyagi, S. Dube, B. J. Poiesz, and F. R. Kramer.** 1999. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc. Natl. Acad. Sci. USA 96:6394–6399.
107. **Wagar, E. A.** 1996. Direct hybridization and amplification applications for the diagnosis of infectious diseases. J. Clin. Lab. Anal. 10:312–325.
108. **Wagner, A., N. Blackstone, P. Cartwright, M. Dick, B. Misof, P. Snow, G. P. Wagner, J. Bartels, M. Murtha, and J. Pendleton.** 1994. Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. Syst. Biol. 43:250–261.
109. **Walsh, P. S., H. A. Erlich, and R. Higuchi.** 1992. Preferential PCR amplification of alleles: mechanisms and solutions. PCR Methods Appl. 1:241–250.
110. **Waters, L. C., S. C. Jacobson, N. Kroutchinina, J. Khandurina, R. S. Foote, and J. M. Ramsey.** 1998. Microchip device for cell lysis, multiplex PCR amplification, and electrophoretic sizing. Anal. Chem. 70:158–162.
111. **Wattel, E., M. Mariotti, F. Agis, E. Gordien, O. Prou, A. M. Courouce, P. Rouger, S. Wain-Hobson, I. S. Chen, and J. J. Lefrere.** 1992. Human T lymphotropic virus (HTLV) type I and II DNA amplification in HTLV-I/II-seropositive blood donors of the French West Indies. J. Infect. Dis. 165:369–372.
112. **Williams, J. F.** 1989. Optimization strategies for the polymerase chain reaction. BioTechniques 7:762–769.
113. **Wilson, I. G.** 1997. Inhibition and facilitation of nucleic acid amplification. App. Environ. Microbiol. 63:3741–3751.
114. **Wilson, W. C., and C. C. L. Chase.** 1993. Nested and multiplex polymerase chain reactions for the identification of bluetongue virus infection in the biting midge, *Culicoides variipennis*. J. Virol. Methods 45:39–47.
115. **Winn-Deen, E. S.** 1996. Automation of molecular genetic methods. Part 2: DNA amplification techniques. J. Clin. Ligand Assay 19:21–26.
116. **Wirz, B., J. D. Tratschin, H. K. Muller, and D. B. Mitchell.** 1993. Detection of hog cholera and differentiation from other pestiviruses by polymerase chain reaction. J. Clin. Microbiol. 31:1148–1154.
117. **Wolcott, M. J.** 1992. Advances in nucleic acid-based detection methods. Clin. Microbiol. Rev. 5:370–386.
118. **Wong, K. C., B. S. W. Ho, S. I. Egglestone, and W. H. P. Lewis.** 1995. Duplex PCR system for simultaneous detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in clinical specimens. J. Clin. Pathol. 48:101–104.
119. **Woo, P. C. Y., S. S. Chiu, W. Seto, and M. Peiris.** 1997. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. J. Clin. Microbiol. 35:1579–1581.
120. **Wu, D. Y., L. Ugozzoli, B. K. Pal, J. Qian, and R. B. Wallace.** 1991. The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell. Biol. 10:233–238.
121. **Yang, C.-F., L. De, B. P. Holloway, M. A. Pallansch, and O. M. Kew.** 1991. Detection and identification of vaccine-related polioviruses by polymerase chain reaction. Virus Res. 20:159–179.
122. **Yourno, J.** 1992. A method for nested PCR with single closed reaction tubes. PCR Methods Appl. 2:60–65.
123. **Zheng, P. S., S. R. Li, T. Iwasaka, J. Song, M. H. Gui, and H. Sugimori.** 1995. Simultaneous detection by consensus multiplex PCR of high- and low-risk and other types of human papilloma virus in clinical samples. Gynecol. Oncol. 58:179–183.
124. **Zimmermann, K., D. Schogl, B. Plaimauer, and J. W. Mannhalter.** 1996. Quantitative multiple competitive PCR of HIV-1 DNA in a single reaction tube. BioTechniques 21:480–484.
125. **Zou, S.** 1997. A practical approach to genetic screening for influenza virus variants. J. Clin. Microbiol. 35:2623–2627.
126. **Zou, S., C. Stansfield, and J. Bridge.** 1998. Identification of new influenza B virus variants by multiplex reverse transcription-PCR and the heteroduplex mobility assay. J. Clin. Microbiol. 36:1544–1548.

ELSEVIER

Molecular and Cellular Probes 18 (2004) 45–50

MOLECULAR AND CELLULAR PROBES

www.elsevier.com/locate/ymcpr

Inhibition of real-time RT–PCR quantification due to tissue-specific contaminants

Ales Tichopad, Andrea Didier, Michael W. Pfaffl*

Institute of Physiology, FML-Weihenstephan, Center of Life and Food Science, Technical University of Munich, Germany

Received 5 March 2003; accepted for publication 5 September 2003

Abstract

Real-time reverse transcription–polymerase chain reaction (RT–PCR) is currently considered the most sensitive method to study low abundance gene expression. Since comparison of gene expression levels in various tissues is often the purpose of an experiment, we studied a tissue-linked effect on nucleic acid amplification. Based on the raw data generated by a LightCycler instrument, we propose a descriptive mathematical model of PCR amplification. This model allowed us to study amplification kinetics of four common housekeeping genes in total RNA samples derived from various bovine tissues. We observed that unknown tissue-specific factors can influence amplification kinetics but this affect can be ameliorated, in part, by appropriate primer selection.

Keywords: Quantitative polymerase chain reaction; Real-time reverse transcription–polymerase chain reaction; Gene expression; Housekeeping genes; Ubiquitin; β-actin; GAPDH; 18S rRNA

1. Introduction

Reverse transcription–polymerase chain reaction (RT–PCR) is the method of choice for quantifying low abundant mRNAs in material such as cells and tissues [1–4]. This method is fast and highly reproducible. Further, its high sensitivity is its principal advantage over other techniques.

In real-time PCR the quantification takes place within an exponential phase of the amplification curve [5]. A crossing point (CP) or threshold cycle (Ct) is then extrapolated to determine a starting amount of template molecules. The CP gives the researcher the first raw information about the expression level of a given gene.

All methods of gene quantification report their findings relative to a measurable base (e.g. copies per cell, weight of tissue, volume of blood, etc.). The correct choice of the denominator depends on the question asked and can significantly affect the quality of the results [6]. To obtain an actual number of copies, various 'absolute' standards are often employed [7–9], but even in these cases, the quantification is always relative as some errors in a protocol are inevitably present [6,10]. So called housekeeping or maintenance genes [11] such as actins, tubulins, albumins, ubiquitin, glyceraldehyd-3-phosphate dehydrogenase (GAPDH), 18S or 28S ribosomal subunits (rRNA) are often used as relative standards [12]. These genes are believed to undergo little, if any, variation in expression under most experimental treatments. Yet, there have been many reports on the regulation of these genes [12–14].

Another important criterion for reliable measurement and comparison of more than one gene is that all of the genes amplify equally. Experiments using normalization with housekeeping genes often overlook this parameter despite the fact that corrections have already be suggested in the literature [15–19].

Many factors present in samples as well as exogenous contaminants have been shown to inhibit PCR (review in Refs. [20,21]). For example, the presence of hemoglobin, fat, glycogen, cell constituents, Ca^{2+}, DNA or RNA concentration, and DNA binding proteins are important factors [20,21]. Additionally, exogenous contaminants such as glove powder and phenolic compounds from

Abbreviations: RT–PCR, reverse transcription–polymerase chain reaction; CP, crossing point; GAPDH, glyceraldehyd-3-phosphate dehydrogenase; FDM, first derivative maximum; SDM, second derivative maximum.

* Corresponding author. Tel.: +49-8161-71-3511; fax: +49-8161-71-4204.

E-mail address: pfaffl@wzw.tum.de (M.W. Pfaffl).

0890-8508/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2003.09.001

the extraction process or the plastic ware can have an inhibiting effect [20,21].

Since some experiments compare gene expression in different organs [9,22], tissue-specific inhibition of DNA amplification may be important. To study the amplification inhibition associated with three randomly chosen tissue types we proposed a mathematical model describing the DNA amplification kinetics in real-time PCR. Using this model we could compare parameters of the amplification kinetics and analyze them statistically.

2. Materials and methods

2.1. Preparation of cDNA samples

Samples of cerebellum, muscle and liver were gathered from six slaughtered cows, immediately frozen in liquid nitrogen and then stored at −80 °C until the total RNA extraction procedure was performed.

Tissue samples were homogenized and total RNA was extracted with a commercially available product, peqGOLD TriFast (Peqlab, Erlangen, Germany), utilizing a single modified liquid separation procedure [23]. No additional purification was performed. Constant amounts of 1000 ng of RNA were reverse-transcribed to cDNA using 200 units of MMLV Reverse Transcriptase (Promega, Mannheim, Germany) according to the manufacturers instructions.

Integrity of the DNA was determined by electrophoresis on 1% agarose gels. Nucleic acid concentrations were measured on a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at $OD_{260\ nm}$ with 220–1600 nm UVettes (Eppendorf). Purity of the RNA extracted was determined as the $OD_{260\ nm}/OD_{280\ nm}$ ratio with expected values between 1.8 and 2.0 (BioPhotometer). A possible trend between the samples and their $OD_{260\ nm}/OD_{280\ nm}$ values was examined.

2.2. Real-time PCR fluorescence data acquisition

Primer sequences of four common housekeeping genes; ubiquitin, β-actin, GAPDH and 18S rRNA were designed to span at least one intron (except for 18S rRNA) and synthesized commercially (MWG Biotech, Ebersberg, Germany) as shown in Table 1. PCR conditions were optimized on a gradient cycler (T-Gradient, Biometra, Göttingen, Germany) and subsequently on a LightCycler (Roche Diagnostic, Mannheim, Germany) [24] by analyzing the melting curves of the products [25]. Real-time PCR using SYBR Green I technology [26] on the LightCycler was then carried out to amplify cDNAs from the tissue samples.

Master-mix for each PCR run was prepared as follows: 6.4 μl of water, 1.2 μl $MgCl_2$ (4 mM), 0.2 μl of each primer (4 pmol), 1.0 μl Fast Start DNA Master SYBR Green I mix (Roche Diagnostics). Finally, 9 μl of master-mix and 25 ng of reverse transcribed total RNA in 1 μl water were transferred into capillaries (end volume 10 μl).

The following amplification program was used: After 10 min of denaturation at 95 °C, 40 cycles of real-time PCR with three-segment amplification were performed with: 15 s at 95 °C for denaturation, 10 s at respective annealing temperature (Table 1) and 20 s at 72 °C for elongation. A melting step was then performed with slow heating starting at 60 °C with a rate of 0.1 °C/s up to 99 °C with continuous measurement of fluorescence. The same gene was always quantified in each run to prevent any inter-run variation.

Fluorescence data from real-time PCR experiments were taken directly from LightCycler software version 3 (Roche Diagnostics), exported to SigmaPlot 2000 (SPSS, Munich, Germany) and fitted with a '*Four-parametric sigmoid model*' as described earlier by our group [27]. Parameters a, b, x_0 and y_0 of each fit were documented together with the coefficient of determination r^2.

All statistics were done in SigmaPlot 2000 (SPSS) and SigmaStat 2.0 (SPSS, Jandel Corporation).

2.3. Crossing point (CP) acquisition

On each individual real-time PCR run, five different CPs were acquired based on different determination procedures. First, the CP was placed into the first derivative maximum ($FDM_{SM} = x_0$) and into the second derivative maximum of the four-parametric sigmoid model (SDM_{SM}) of each run as shown earlier [27].

Table 1
Details of primers used to amplify four housekeeping genes

Gene	Primers	Sequence length (bp)	Annealing temperature (°C)
Ubiquitin	for: AGA TTC AGG ATA AGG AAG GCA T rev: GCT CCA CCT CCA GGG TGA T	198	60
GAPDH	for: GTC TTC ACT ACC ATG GAG AAG G rev: TCA TGG ATG ACC TTG GCC AG	197	58
18S rRNA	for: GAG AAA CGG CTA CCA CAT CCA A rev: GAC ACT CAG CTA AGA GCA TCG A	338	60
β-actin	for: AAC TCC ATC ATG AAG TGT GAC G rev: GAT CCA CAT CTG CTG GAA GG	234	60

Table 2
Two-way ANOVA

Factor	a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}
Tissue	0.01	<0.001	0.004	0.02	0.005	0.008	0.004
Gene	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Tissue–gene interaction	0.004	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Further, CP was computed using the '*Fit point method*' (FP_{LC}) [5] and '*Second derivative maximum method*' (SDM_{LC}) [5,28], both part of the LightCycler software 3.3 (Roche Diagnostics). In the FP_{LC} method, uninformative background fluorescence observations were discarded by setting a constant noise band. An intersecting line was then arbitrarily placed at the base of the exponential portion of the amplification curves. This generated CPs acquired at a constant fluorescence level (value 2 in our case).

In the SDM_{LC} method the second derivative maximum is calculated by LightCycler software based on an unknown and unpublished mathematical approximation of partial amplification kinetics around the supposed SDM_{LC} [5,28]. The FP_{LC} and SDM_{LC} were directly obtained from the calculated values by the LightCycler software 3.3 (Roche Diagnostics).

Eventually, the '*Taqman threshold level*' (Ct) or CP [29] computing method was simulated by fitting the intersecting line upon the 10 times value of ground fluorescence standard deviation (CP_{Tm}). In the '*Taqman threshold level*' procedure, the y_0 values of the four-parametric sigmoid model were considered ground fluorescence.

While parameters a and b describe amplification kinetics, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, and CP_{Tm} are considered quantification parameters since they are clearly defined constants within the model.

Table 3a
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of ubiquitin amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	43.118	1.950	25.649	23.082	20.180	21.817	22.680	1.000
	CV (%)	9.56	1.17	1.31	1.50	2.13	1.43	1.77	0.004
Liver	Mean	39.355	2.004	26.184	23.545	20.688	22.288	22.597	1.000
	CV (%)	7.79	1.62	1.43	1.64	1.73	1.79	1.47	0.010
Muscle	Mean	41.958	2.064	26.443	23.725	20.637	22.487	25.370	0.999
	CV (%)	5.40	2.25	0.81	0.94	1.52	1.15	0.67	0.018
$Mean_{total}$		41.477	2.006	26.092	23.450	20.502	22.197	23.549	1.000
$CV_{in\text{-}tissue}$ (%)		7.58	1.68	1.18	1.36	1.79	1.46	1.30	0.011
$CV_{out\text{-}tissue}$ (%)		4.65	2.85	1.55	1.41	1.36	1.55	6.70	0.014

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Table 3b
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of GAPDH amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	47.223	2.075	23.663	20.930	18.185	19.583	20.483	0.998
	CV (%)	11.48	1.43	1.14	1.36	1.90	1.43	2.01	0.009
Liver	Mean	46.675	2.094	24.936	22.179	19.322	20.868	21.580	0.998
	CV (%)	6.39	2.75	1.61	1.97	2.09	2.21	2.20	0.020
Muscle	Mean	52.415	2.228	21.588	18.653	15.800	17.440	16.377	0.997
	CV (%)	3.79	2.94	3.30	4.08	4.70	4.06	4.48	0.032
$Mean_{total}$		48.771	2.132	23.396	20.587	17.769	19.297	19.480	0.998
$CV_{in\text{-}tissue}$ (%)		7.22	2.37	2.02	2.47	2.90	2.57	2.89	0.020
$CV_{out\text{-}tissue}$ (%)		6.50	3.92	7.22	8.68	10.12	8.98	14.08	0.068

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Table 3c
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of 18S rRNA amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	49.782	2.701	15.274	11.717	9.518	10.556	10.923	0.996
	CV (%)	3.76	5.33	3.64	6.26	6.32	5.83	6.17	0.047
Liver	Mean	53.544	2.897	14.669	10.854	8.638	9.809	9.185	0.996
	CV (%)	3.35	2.55	9.01	12.21	12.28	12.38	11.15	0.040
Muscle	Mean	55.943	2.752	15.369	11.744	9.250	10.573	10.267	0.997
	CV (%)	2.67	2.31	5.61	7.76	8.32	7.94	7.75	0.041
$Mean_{total}$		53.090	2.784	15.104	11.439	9.135	10.313	10.125	0.996
$CV_{in\text{-}tissue}$ (%)		3.26	3.40	6.09	8.74	8.97	8.72	8.36	0.042
$CV_{out\text{-}tissue}$ (%)		5.85	3.66	2.51	4.43	4.94	4.23	8.67	0.019

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

2.4. Statistical evaluation of model parameters

Two-way ANOVA with tissue as the first factor of three levels (cerebellum, muscle and liver) and gene as the second factor of four levels (ubiquitin, β-actin, GAPDH, 18S rRNA) was applied to the parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC} and CP_{Tm} (Table 2). Normal distribution was given within the data sets.

For all above-mentioned parameters and r^2 following statistical indicators were calculated (Tables 3a–3d)

- Interaction mean (i.e. from the six values within one level of factor gene and one level of factor tissue) and interaction coefficient of variance-CV.
- Total mean ($mean_{total}$) out of 18 values (always six samples in three tissues) for each factor gene.
- Mean value out of three CVs ($CV_{in\text{-}tissue}$) reporting internal variance within all three tissue levels.
- Coefficient of variance out of three interaction means ($CV_{out\text{-}tissue}$) showing a variability caused by factor tissue.

3. Results and discussion

All primers used could satisfactorily amplify the flanked sequence. The melting curve analysis and gel analysis detected very little, if any, nonspecific product. We approximated the PCR amplification kinetics with the four-parametric sigmoid model. This model describes well (in all data sets $r^2 > 0.99, n = 40$) the entire fluorescence curve and therefore its beginning and end do not need to be arbitrarily delimited [19]. Nevertheless, correlation between values of b and r^2 showed that there were differences in the goodness of the fit (Pearson correlation coefficient $r = 0.915, n = 72$). The best fit was in runs with high amplification efficiencies. With decreasing amplification efficiency the determination power of the model also decreased.

There is an integral purification step at the end of the extraction procedure [23], consisting of repeated washing the final total RNA pellet with ethanol. In this study no additional RNA purification was performed since additional purification decreases yield and is often omitted. This procedure simulated a routine PCR sample preparation as it is carried out in most labs. The contamination within the RNA samples detected as $OD_{260\ nm}/OD_{280\ nm}$ ratios was not significantly related to the type of tissue (data not shown).

Statistical analysis of the parameters a and b (Table 2) under an influence of the two experimental factors showed that the tissue was the largest source of variance and the primer sequences had the least affect [21,22].

A similar trend of variability within the log-linear trajectory slope (b) and plateau height (a) showed that the tissue from

Table 3d
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of β-actin amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	85.015	1.418	22.499	20.632	16.640	19.362	19.643	1.000
	CV (%)	5.11	2.15	2.22	2.54	3.21	2.69	2.34	0.004
Liver	Mean	86.694	1.467	23.555	21.624	17.400	20.348	18.633	1.000
	CV (%)	2.14	1.31	0.85	0.95	1.52	1.11	1.11	0.002
Muscle	Mean	84.886	1.470	24.264	22.328	18.230	21.047	20.813	1.000
	CV (%)	2.75	3.53	0.90	1.03	1.14	1.16	0.88	0.005
$Mean_{total}$		85.532	1.452	23.440	21.528	17.423	20.252	19.697	1.000
$CV_{in\text{-}tissue}$ (%)		1.00	2.33	1.32	1.51	1.96	1.65	1.45	0.004
$CV_{out\text{-}tissue}$ (%)		1.18	2.01	3.79	3.96	4.56	4.18	5.54	0.001

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

which total RNA was extracted has a significant effect on the PCR kinetics and thus on the CP acquisition (Table 2). This can be caused by different amounts of cellular debris present in samples after RNA extraction [30,31]. Also endogenous contaminants such as blood or fat play an important role. Contamination of the sample may affect both the PCR as well as the preceding RT reaction [20,21].

Since interaction between both factors; tissue and gene is significant, the tissue-specific disturbance is not the same for all four amplified sequences but rather is sequence-specific. In our study, the highest resistance to tissue-specific disturbance showed the sequence of β-actin followed by ubiquitin, 18S rRNA and GAPDH (see $CV_{out\text{-}tissue}$ values in Tables 3a–3d). A plausible explanation of this interaction may be the presence of specific DNA blocking by polysaccharides or proteins present as endogenous contaminants in the sample [32]. It is possible that DNA amplification may be affected by regions of the template DNA that are specifically blocked by these endogenous macromolecules. Our data show that not only the choice of housekeeping genes [12–14] but also tissue-specific factors and the sequence-specific factors can affect the expression assays.

Tissue-specific suppression can be compensated, in part, by well performing primers such as those for β-actin and ubiquitin used here. From this data it seems that sequences that amplified with higher efficiency (i.e. small *b*) better resist inhibition and show lower variance in all parameters of the PCR kinetics (compare $mean_{total}$ of *b* and $CV_{out\text{-}tissue}$ values in Tables 3a and 3d with Tables 3b and 3c). Thus, primer selection and documenting the reaction efficiency are important PCR optimization steps. Although housekeeping genes are expressed differently in various tissues our data show that some vary less than others. For example, ubiquitin showed marginally higher variance between tissues than within one tissue (compare $CV_{out\text{-}group}$ with $CV_{in\text{-}group}$ in Table 3a). This suggests that the expression of ubiquitin in the different tissues was similar. The low variance for ubiquitin expression between tissues suggests that it is the best standard but is closely followed by β-actin and GAPDH. 18S rRNA, with its high variance, seems to be less suitable as an internal standard. This order was preserved in all CP computing methods.

Each method of computing CPs seems to be accurate for estimating expression levels but they varied slightly when CP acquisitions took place at different heights of the amplification curve (Tables 3a–3d). The method of first and second derivative maximum computed from the four-parametric sigmoid model is reliable and simple and generates reliable CPs comparable with other methods (see CV values in Tables 3a–3d).

Acknowledgements

The experimental animals were slaughtered at the EU-official slaughterhouse of the Bayerische Landesanstalt für Tierzucht at Grub, 85580 Poing, Germany.

References

[1] Schmittgen TD. Real-time quantitative PCR. Methods 2001;25:383–5.
[2] Orlando C, Pinzani P, Pazzagli M. Developments in quantitative PCR. Clinical Chemistry and Laboratory Medicin 1998;36:255–69.
[3] Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT–PCR. Genome Research 1996;6:995–1001.
[4] Freeman WM, Walker SJ, Vrana KE. Quantitative RT–PCR: pitfalls and potential. BioTechniques 1999;26:112–25.
[5] Rasmussen R. Quantification on the lightcycler instrument. In: Meuer S, Wittwer C, Nakagawara K, editors. Rapid cycle real-time PCR: methods and applications. Heidelberg: Springer; 2001. p. 21–34.
[6] Ferré F. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods and Applications 1992;2:1–9.
[7] Pfaffl MW, Hageleit M. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT–CR. Biotechnology Letters 2001;23:275–82.
[8] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 2000;25:169–93.
[9] Tichopad A, Pfaffl MW, Didier A. Tissue-specific expression pattern of bovine prion: quantification using real-time RT–PCR. Molecular and Cellular Probes 2003;17:5–10.
[10] Souazé F, Ntodou-Thomé A, Tran CY, Rostene W, Forgez P. Quantitative RT–PCR: limits and accuracy. BioTechniques 1996;21:280–5.
[11] Warrington JA, Nair A, Mahadevappa M, Tsygantskaya M. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiological Genomics 2000;2:143–7.
[12] Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E. Housekeeping genes as internal standards: use and limits. Journal of Biotechnology 1999;75:291–5.
[13] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT–PCR. Journal of Biochemical and Biophysical Methods 2000;20:69–81.
[14] Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. BioTechniques 2000;29:332–7.
[15] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 2001;25:402–8.
[16] Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research 2001;29(9):e45.
[17] Pfaffl MW, Horgan GW, Dempfle L. Relative Expression Software Tool (REST©) for group wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 2002;30(9):e36.
[18] Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. Journal of Molecular Diagnostics 2001;3:55–61.
[19] Weihong L, Saint A. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Analytical Biochemistry 2002;302:52–9.
[20] Wilson IG. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 1997;63:3741–51.
[21] Rossen L, Norskov P, Holmstrom K, Rasmussen FO. Inhibition of PCR by components of food sample, microbial diagnostic assay and DNA-extraction solutions. International Journal of Food Microbiology 1992;17:37–45.
[22] Pfaffl MW, Lange IG, Daxenberger A, Meyer HH. Tissue-specific expression pattern of estrogen receptors (ER): quantification of ER

alpha and ER beta mRNA with real-time RT–PCR. Acta Pathologica Microbiologica et Immunologica Scandinavica 2001; 109:345–55.

[23] Chomczynski PA. Reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 1993;15:532–4.

[24] Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The lightcycler: a microvolume multisample fluorimeter with rapid temperature control. BioTechniques 1997;22:176–81.

[25] Ririe KM, Rasmussen RT, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry 1997;245:154–60.

[26] Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. BioTechniques 1998;24:954–8.

[27] Tichopad A, Dzidic A, Pfaffl MW. Improving quantitative real-time RT–PCR reproducibility by boosting primer-linked amplification efficiency. Biotechnology Letters 2002;24:2053–6.

[28] Wittwer CT, Gutekunst M, Lohmann S. Method for quantification of an analyte. United States Patent No. US, 6,303,305 B1

[29] Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proceedings of the National Academy of Science of the United States of America 1991;15:7276–80.

[30] Wilson IG, Gilmour A, Cooper JE. Detection of toxigenic microorganisms in foods by PCR. In: Kroll RG, Gilmour A, Sussman M, editors. In new techniques in food and beverage microbiology. London: Blackwell; 1993. p. 163–72.

[31] Starnbach MN, Falkow S, Tomkins SL. Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization. Journal of Clinical Microbiology 1989;27:1257–61.

[32] Rijpens NP, Jannes G, Van Asbroeck M, Rossau R, Herman LMF. Direct detection of *Brucella* spp. In raw milk by PCR and reverse hybridization with 16S–23S rRNA spacer probes. Applied Environmental Microbiology 1996;62:1683–8.

African Journal of Biotechnology Vol. 2 (5), pp. 91-95, May 2003
Available online at www.academicjournals.org/AJB
ISSN 1684-5315 © 2003 Academic Journals

Minireview

Bioinformatic tools and guideline for PCR primer design

Kamel A. Abd-Elsalam

Molecular Markers Lab., Plant Pathology Research Institute, Agricultural Research Center, Orman 12619, Giza, Egypt

Accepted 28 April 2003

Bioinformatics has become an essential tool not only for basic research but also for applied research in biotechnology and biomedical sciences. Optimal primer sequence and appropriate primer concentration are essential for maximal specificity and efficiency of PCR. A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. There are several online tools devoted to serving molecular biologist design effective PCR primers. This review intends to provide a guide to choosing the most efficient way to design a new specific-primer by applying current publicly available links and Web services. Also, the purpose here is to provide general recommendations for the design and use of PCR primers.

Key words: Bio-computing, primer design, web-based resources.

INTRODUCTION

In the last 10 to 15 years the computer has become an essential companion for cell and molecular biologists. Bioinformatics is an emerging scientific discipline that uses information technology to organize, analyze, and distribute biological information in order to answer complex biological questions. Bioinformatics is an interdisciplinary research area, which may be broadly defined as the interface between biological and computational sciences (Singh and Kumar, 2001). It involves the solution of complex biological problems using computational tools and systems. It also includes the collection, organization, storage and retrieval of biological information from databases. Selection of oligonucleotide primers is useful for polymerase chain reaction (PCR), oligo hybridization and DNA sequencing. Proper primer design is actually one of the most important factors/steps in successful DNA sequencing. Various bioinformatics programs are available for selection of primer pairs from a template sequence. The plethora programs for PCR primer design reflects the central role of PCR in modem molecular biology. Nevertheless, all these computer programs are written mainly to assist in the primer design process and are not meant to replace the eye of the experienced researcher, especially considering the sometimes erratic nature of PCR experiments. When scheduling important PCR experiments, it is usually worthwhile to evaluate the predictions of numerous different programs and to use common sense and laboratory experience to evaluate the suggested primers before committing to their synthesis (Binas, 2000). This review summarizes the general guidelines for primer design online.

Corresponding author; Tel: 002 02 5724893, Fax: 002 02 4723146, E-mail: kaabdelsalam@msn.com

WEB-BASED RESOURCES FOR PRIMER DESIGN

There are a numerous web-based resources for PCR and primer design. Though most are freely available, they are of variable quality and not well maintained. This often results in missing links and so sites that may have been useful previously may not be functional at a later date. There are a number of criteria that need to be established in the design of primers and a number of these are listed below (Tables 1and 2).

SOFTWARE IN PRIMER DESIGN

The use of software in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Freeware software is available on the internet and many universities have established servers where a user can log on and perform free analyses of proteins and nucleic acid sequences (Singh and Kumar, 2001). There are number of simple stand-alone programs as well as complex integrated networked versions of the commercial software available. These software packages may be for complete DNA and protein analysis, secondary structure predictions, primer design, molecular modeling, development of cloning strategies, plasmid drawing or restriction enzyme analyses. Companies engaged in biosoftware development include: Alkami Biosystems, Molecular Biology Insights, PREMIER Biosoft International, IntelliGenetics Inc., Hitachi Inc., DNA Star, Advanced American Biotechnology and Imaging. Some scientists have also developed algorithms and computer programs for various purposes of primer design (Rychlik and Rhoades, 1989; Lowe et al., 1990; Lucas et al., 1991; O'Hara and Venezia, 1991; Tamura et

Table 1. Online primer design sites.

Tool name	Description	www
CODEHOP	Consensus Degenerate Hybrid Oligonucleotide Primers; degenerate PCR primer design; will accept unaligned sequences.	http://blocks.fhcrc.org/codehop.html
Gene Fisher	Interactive primer design tool for standard or degenerate primers; will accept unaligned sequences.	http://bibiserv.techfak.uni-bielefeld.de/genefisher/
DoPrimer	Easily design primers for PCR and DNA sequencing.	http://doprimer.interactiva.de/
Primer3	Comprehensive PCR primer and hybridization probe design tool; many options but easy to accept defaults at first.	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi http://www.basic.nwu.edu/biotools/Primer3.html http://www.justbio.com/primer/index.php
Primer Selection	Select PCR primers from nucleotide sequence.	http://alces.med.umn.edu/rawprimer.html
Web Primer	Allow alternative design of primers for either PCR or sequencing purpose.	http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer
PCR Designer	For restriction analysis of sequence mutations.	http://cedar.genetics.soton.ac.uk/public_html/primer.html
Primo Pro 3.4	Reduces PCR noise by lowering the probability of random primering.	http://www.changbioscience.com/primo/primo.html
Primo Degenerate 3.4	Primo Degenerate 3.4 designs PCR primers based on a single peptide sequence or multiple alignments of proteins or nucleotides.	http://www.changbioscience.com/primo/primod.html
PCR Primer Design	An application that designs primers for PCR or sequencing purposes.	http://pga.mgh.harvard.edu/servlet/org.mgh.proteome.Primer
The Primer Generator	The program analyzes the original nucleotide sequence and desired amino acid sequence and designs a primer that either has a new restriction enzyme site or is missing an old one.	http://www.med.jhu.edu/medcenter/primer/primer.cgi
EPRIMER3	Picks PCR primers and hybridization oligos (EMBOSS).	http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html
PRIMO	Prediction of forward and reverse oligonucleotide Primers.	http://bioweb.pasteur.fr/seqanal/interfaces/primo.html3 http://atlas.swmed.edu/primo/primo_form.html
PrimerQuest	A primer design tool.	http://www.idtdna.com/biotools/primer_quest/primer_quest.asp
MethPrimer	Design primers for methylation PCRs.	http://itsa.ucsf.edu/~urolab/methprimer/index1.html
Rawprimer	A tool for selection of PCR primers.	http://alces.med.umn.edu/rawprimer.html
MEDUSA	A tool for automatic selection and visual assessment of PCR primer pairs.	http://www.cgr.ki.se/cgr/MEDUSA/
The Primer Prim'er Project	Software suite that completely automates the PCR primer design process.	http://www-nmr.cabm.rutgers.edu/bioinformatics/Primer_Primer_Project/Primer.html
Oligonucleotides for the PCR	Seek oligonucleotides on both sides of an area.	http://www.citi2.fr/bio2/Oligo2lib.html
GAP	Genome- wide Automated Primer finder servers.	http://promoter.ics.uci.edu/Primers/

al., 1991; Makarova et al., 1992; Osborne, 1992; Li et al.,1997; Plasterer, 1997; Sze et al., 1998; Gorelenkov et al., 2001). Many programs aiding in the design of primers exist (Table 3).

GUIDELINES FOR THE DESIGN AND USE OF PRIMERS

DNA Template and oligonucleotide primers must be considered in greater detail (Linz et al., 1990). Efficacy and sensitivity of PCR largely depend on the efficiency of primers (He et al., 1994). The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors including: a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures; b) duplex stability of mismatched nucleotides and their location; and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. The primers which are unique for the target sequence to be amplified should fulfill certain criteria such as primer length, GC%, annealing and melting temperature, 5' end stability, 3' end specificity etc (Dieffenbach et al., 1995). Most of the

Table 2. PCR oligonucleotide resources.

Oligonucléotides pour la PCR	Calculation of melting point of a oligonucleotide.	http://www.citi2.fr/bio2/OligoTM.html
Oligonucleotide properties calculator	Prediction of melting temperature.	http://www.basic.nwu.edu/biotools/oligocalc.html http://www.microbiology.adelaide.edu.au/learn/oligcalc.htm
Oligonucleotide analyzer	Generates Tm, free energy, molecular weight and hairpin and dimer formation structures.	http://www.nature.com/oligonucleotide.html
Oligo Tm Determination	Prediction of Tm.	http://alces.med.umn.edu/rawtm.html
Poland	Prediction of melting temperatures of primers.	http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html
PROLIGO	Oligos parameter calculation.	http://www.gensetoligos.com/Calculation/calculation.html

Table 3. PCR primers design software for personal computer.

Software name	Descreption	www
PrimerSelect	Analyzes a template DNA sequence and chooses primer pairs for PCR and primers for DNA sequencing.	www.dnastar.com
DNASIS Max	DNASIS Max is a fully integrated program that includes a wide range of standard sequence analysis features.	http://www.medprobe.com/no/dnasis.html
Primer Premier 5	primer design for Windows and Power Macintosh.	http://www.premierbiosoft.com/primerdesign/primerdesign.html
Primer Premier:	Comprehensive primer design for Windows and Power Macintosh.	http://www.premierbiosoft.com/
NetPrimer	Comprehensive analysis of individual primers and primer pairs.	http://www.premierbiosoft.com/NetPrimer.html
Array Designer 2	For fast, effective design of specific oligos or PCR primer pairs for microarrays	http://www.premierbiosoft.com/dnamicroarray/dnamicroarray.html
Beacon Designer 2.1	Design molecular beacons and TaqMan probes for robust amplification and fluorescence in real time PCR.	http://www.premierbiosoft.com/molecular_beacons/taqman_molecular_beacons.html
GenomePRIDE 1.0	Primer design for DNA-arrays/chips.	http://pride.molgen.mpg.de/genomepride.html
Fast PCR	Software for Microsoft Windows has specific, ready-to-use templates for many PCR and sequencing applications: standard and long PCR, inverse PCR, degenerate PCR directly on amino acid sequence, multiplex PCR.	http://www.biocenter.helsinki.fi/bi/bare-1_html/manual.htm
OLIGO 6	Primer Analysis Software for Mac and Windows.	http://www.oligo.net/
Primer Designer 4	Will find optimal primers in target regions of DNA or protein molecules, amplify features in a molecule, or create products of a specified length.	http://www.scied.com/ses_pd5.htm
GPRIME	Software for primer design.	http://life.anu.edu.au/molecular/software/gprime.htm
Sarani Gold	Genome Oligo Designer is software for automatic large-scale design of optimal oligonucleotide probes for microarray experiments.	http://mail.strandgenomics.com/products/sarani/
PCR Help	Primer and template design and analysis	http://www.techne.com/CatMol/pcrhelp.htm
Genorama chip Design Software	Genorama Chip Design Software is complete set of programs required for genotyping chip design. The programs can also be bought separately.	http://www.asperbio.com/Chip_desin_soft.htm
Primer Designer	The Primer Designer features a powerful, yet extremely simple, real-time interface to allow the rapid identification of theoretical ideal primers for your PCR reactions.	http://genamics.com/expression/primer.htm
Primer Premier	Automatic design tools for PCR, sequencing or hybridization probes, degenerate primer design, Nested/Multiplex primer design, restriction enzyme analysis and more.	http://www.biotechniques.com/freesamples/itembtn21.html
PrimerDesign	DOS-program to choose primer for PCR or oligonucleotide probes.	http://www.chemie.unimarburg.de/%7Ebecker/pdhome.html

reviews on PCR optimization (Erlich et al., 1991; Dieffenbach et al., 1995; Roux, 1995) consider different parameters of PCR but generally do not discuss basic concepts of PCR primer design.

Maybe the most critical parameter for successful PCR is the design of Primers. All things being equal, a poorly designed primer can result in a PCR reaction that will not work. The primer sequence determines several things such as the length of the product, its melting temperature and ultimately the yield. A badly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. This application note is provided to give rules that should be taken into account when designing primers for PCR. More comprehensive coverage of this subject can be found elsewhere (Dieffenbach et al., 1995).

The sequences of the primers used for PCR amplification can have a major effect on the specificity and sensitivity of the reaction. When choosing two PCR amplification primers, the following guidelines should be considered:

Primer length: Since both specificity and the temperature and time of annealing are at least partly dependent on primer length, this parameter is critical for successful PCR (Wu et al., 1991). For broad-spectrum studies, primers of typically 18-30 nucleotides in length are the best. Primers should be at least 18 nucleotides in length to minimize the chances of encountering problems with a secondary hybridization site on the vector or insert. Primers with long runs of a single base should generally be avoided. It is especially important to avoid 4 or more G's or C's in a row.

Melting Temperature (T_m): The optimal melting temperatures for primers in the range 52-58°C, generally produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65°C should also be avoided because of potential for secondary annealing. It is then advisable to do the sequencing reaction with annealing and extension at 60°C. A good working approximation of this value (generally valid for oligos in the 18–30 base range) can be calculated using the formula of Wallace et al. (1979), $T_m = 2(A+T) + 4(G+C)$. Using improved nearest-neighbor thermodynamic values given by SantaLucia et al. (1996), an estimate of melting temperature can be obtained for oligonucleotide analysis.

GC Content (Tm and Ta are Interrelated): GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have a GC content between 45 and 60 percent (Dieffenbach et al., 1995). For primers with a G/C content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50°C. GC content, melting temperature and annealing temperature are strictly dependent on one another (Rychlik et al., 1990).

3'-End Sequence: It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming (Kwok et al., 1990). Primers should be "stickier" on their 5' ends than on their 3' ends. A "sticky" 3' end as indicated by a high G/C content could potentially anneal at multiple sites on the template DNA. A "G" or "C" is desirable at the 3' end but the first part of this rule should apply. This GC clamp reduces spurious secondary bands (Sheffield et al., 1989).

Dimers and false priming cause misleading results: Primers should not contain complementary (palindromes) within themselves; that is, they should not form hairpins. If this state exists, a primer will fold back on itself and result in an unproductive priming event that decreases the overall signal obtained (Breslauer et al., 1986). Hairpins that form below 50°C generally are not such a problem. Primers should not contain sequences of nucleotides that would allow one primer molecule to anneal to itself or to the other primer used in PCR reactions (primer dimer formation).

Specificity: As mentioned above, primer specificity is at least partly dependent on primer length. It is evident that there are many more unique 24 base oligos than there are 15 base pair oligos. However, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.

Degenerate Primers: Degeneracy in primer sequence should also be taken into consideration. Degenerate primers based on the amino acid sequence of conserved regions were also used to search for members of a gene family (Wilks et al., 1989). Computer programs have also been developed specifically for degenerate primer design (Chen and Zhu, 1997).

Complementary primer sequences: Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back" can occur. Another related danger is inter-primer homology: partial homology in the middle regions of two primers can interfere with hybridization. If the homology should occur at the 3' end of either primer, primer dimer formation will occur.

Other recommendations: The concentration of primer in amplification reaction should be between 0.1 and 0.5 µm. If possible, a computer search should be conducted against the vector and insert DNA sequences to verify that the primer and especially the 8-10 bases of its 3' end

are unique. Inosine should not be included in sequencing primers. They either do not work or give poor cycle sequencing results. The design of PCR and DNA sequencing primers follows very similar guidelines. Even though primer characteristics can be visually inspected for the presence of the elements listed above, a number of computer programs that have been developed use several of these guidelines for primer selection.

CONCLUSION

The key to the PCR lies in the design of the two oligonucleotide primers. It is essential that care is taken in the design of primers for PCR. Several parameters including the length of the primer, %GC content and the 3' sequence need to be optimized for successful PCR. Certain of these parameters can be easily by hand optimized while others are best done with marketable computer programs. The increasing use of information from the internet and the sequences held in gene databases are practical starting points when designing primers and reaction conditions for the PCR. A number or software packages such as Oligo, Primer etc. have allowed the process of primer design to be less troublesome. It is also possible to include more than one set of primers in a PCR.

REFERENCES

Binas M (2000). Designing PCR primers on the web. Biotechniques 29: 988-990.

Breslauer KJ, Ronald F, Blocker H, Marky LA (1986). Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. 83: 3746-3750.

Chen H, Zhu G (1997). Computer program for calculating the melting temperature of degenerate oligonucleotides used in PCR or hybridization. Biotechniques 22: 1158-1160

Dieffenbach CW, Lowe TMJ, Dveksler GS (1995). General Concepts for PCR Primer Design. In: PCR Primer, A Laboratory Manual, Dieffenbach CW, Dveksler GS Ed., Cold Spring Harbor Laboratory Press, New York, 133-155.

Erlich HA, Gelfand D, Sninsky JJ (1991). Recent advances in the polymerase chain reaction. Science 252: 1643-1651.

Gorelenkov V, Antipov A, Lejnine S, Darasella N, Yuryev A (2001). Set of novel tools for PCR primer design. Biotechniques 31:1326-1330.

He Q, Marjamaki M, Soini H, Mertsola J, Viljanen MK (1994). Primers are decisive for sensitivity of PCR. Biotechniques. 17: 82-87.

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky J (1990). Effects of primer-template mismatches on the polymerase chain reaction: Human Immunodeficiency Virus 1 model studies. Nucleic Acids Res. 18: 999-1005.

Li PKC, Kupfer CJ, Davies D, Burbee G, Evans A, Garner HR (1997). PRIMO: A primer design program that applies base quality statistics for automated large-scale DNA sequencing. Genomics 40:476-485.

Linz U, Delling U, Rubsamem-Waigmann (1990). Systematic studies on parameters influencing the performance of the polymerase chain reaction. J. Clin. Chem. Clin. Biochem. 28: 5-13.

Lowe T, Sharefkin J, Yang SQ, Dieffenbach CW (1990). A computer program for selection of oligonucleotide primers for polymerase chain reaction. Nucleic Acids Res. 18: 1757-1761.

Lucas K, Busch M, Mossinger S, Thompson JA (1991). An improved microcomputer program for finding gene or gene family-specific oligonucleotides suitable as primers for polymerase chain reactions or as probes. Comput. Appl. Biosci. 7: 525-529.

Makarova KS, Mazin AV, Wolf YI, Soloviev VV (1992). DIROM: An experimental design interactive system for directed mutagenesis and nucleic acid engineering. Comput. Appl. Biosci. 8: 425-431.

O'Hara PJ, Venezia D (1991). PRIMEGEN: a tool for designing primers from multiple alignments. CABIOS 7: 533-534.

Osborne BI (1992). Hyper PCR: A Macintosh Hypercard program for determination of optimal PCR annealing temperature. Comput. Appl. Biosci. 8: 33-34.

Plasterer TN (1997). PRIMERSELECT: Primer and probe design. Methods Mol. Biol. 70: 291-302.

Roux KH (1995). Optimization and troubleshooting in PCR in: PCR methods and applications. Cold spring Harbor laboratory. 5185-5194.

Rychlik W, Rhoads RE (1989). A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. Nucleic Acids Res. 17: 8543-8551.

Rychlik W, Spencer WJ, Rhoads RE (1990). Optimisation of annealing temperature for DNA amplification in vitro. Nucleic Acids Res. 18: 6409-6412.

SantaLucia JJr, Allawi HAT, Seneviratne PA (1996). Improved Nearest-Neighbor parameters for predicting DNA duplex stability. Biochemistry 35: 3555-3562.

Sheffield VC, Cox DR, Lerman LS, Myers RM (1989). Attachment of a 40 base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single base changes. Proc. Natl. Acad. Sci. 86: 232-236.

Singh VK, Kumar A (2001). PCR Primer Design. Mol. Biol. Today 2: 27-32.

Sze SH, Raytberg MA, Gelfand MS, Mironov AA, Astakhova TV, Pevzner PA (1998). Algorithms and software for support of gene identification experiments. Bioinformatics 14: 14-19.

Tamura T, Holbrook SR, Kim SH (1991). A Macintosh computer program for designing DNA sequences that for specific peptides and proteins. Biotechniques 10: 782-784.

Wallace RB, Shaffer J, Murphy RF, Bonner J, Hirose T, Itakura K (1979). Hybridization of synthetic oligodeoxyribonucleotides to phi chi 174 DNA: the effect of single base pair mismatch. Nucleic Acids Res. 6: 3543-3557.

Wilks AF, Kurban RR, Hovens CM, Ralph SJ (1989). The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. Gene 85: 67-74.

Wu DYW, Ugozzoli L, Pal BK, Qian J, Wallace RB (1991). The effect of temperature and oligonucleotide primer length on specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell Biol. 10: 233-238.